

Genome-wide Expression Profiling of the Response to Polyene, Pyrimidine, Azole, and Echinocandin Antifungal Agents in *Saccharomyces cerevisiae**[§]

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Antifungal compounds exert their activity through a variety of mechanisms, some of which are poorly understood. Novel approaches to characterize the mechanism of action of antifungal agents will be of great use in the antifungal drug development process. The aim of the present study was to investigate the changes in the gene expression profile of *Saccharomyces cerevisiae* following exposure to representatives of the four currently available classes of antifungal agents used in the management of systemic fungal infections. Microarray analysis indicated differential expression of 0.8, 4.1, 3.0, and 2.6% of the genes represented on the Affymetrix S98 yeast gene array in response to ketoconazole, amphotericin B, caspofungin, and 5-fluorocytosine (5-FC), respectively. Quantitative real time reverse transcriptase-PCR was used to confirm the microarray analyses. Genes responsive to ketoconazole, caspofungin, and 5-FC were indicative of the drug-specific effects. Ketoconazole exposure primarily affected genes involved in ergosterol biosynthesis and sterol uptake; caspofungin exposure affected genes involved in cell wall integrity; and 5-FC affected genes involved in DNA and protein synthesis, DNA damage repair, and cell cycle control. In contrast, amphotericin B elicited changes in gene expression reflecting cell stress, membrane reconstruction, transport, phosphate uptake, and cell wall integrity. Genes with the greatest specificity for a particular drug were grouped together as drug-specific genes, whereas genes with a lack of drug specificity were also identified. Taken together, these data shed new light on the mechanisms of action of these classes of antifungal agents and demonstrate the potential utility of gene expression profiling in antifungal drug development.

Invasive fungal infections cause significant morbidity and mortality in susceptible patient populations (1–4). Currently available antifungal agents are few and have limitations with regard to efficacy and toxicity (5). The need for alternative strategies in the management of fungal infections has increased with the emergence of new fungal pathogens and the development of antifungal resistance (6, 7). Novel approaches to identify unique antifungal drug targets are needed. Furthermore, a greater understanding of the mechanisms of action of available antifungal agents would aid in this effort.

Currently only four classes of antifungal agents are used to treat invasive fungal disease. Amphotericin B, the only polyene effective systemically, has been in use for over 40 years. It exerts its activity by binding to ergosterol in the fungal cell membrane, compromising membrane integrity, and ultimately leading to cell death (8, 9). The antimetabolite, 5-fluorocytosine (5-FC),¹ acts by interfering with nucleotide metabolism and inhibiting RNA, DNA, and protein synthesis (10). The more recently introduced azole antifungal agents exert their activity by inhibiting the biosynthesis of ergosterol, the major membrane sterol of fungi. The azoles inhibit the cytochrome P450 enzyme, lanosterol demethylase, by binding to the heme in the active site of the enzyme (6, 11). The newest class of antifungal agents used in the management of systemic fungal infections is the echinocandins. Caspofungin is currently the only commercially available agent in this class. It exerts its activity by inhibiting the synthesis of β -1,3-glucan in the fungal cell wall (12).

One of the ways in which cells adjust to changes in their environment is by altering gene expression patterns. Thus, measurement of changes in gene expression upon exposure to a drug can help determine how drugs and drug candidates work in cells and organisms. DNA microarray technology is a powerful tool to measure gene expression on a genomic scale, allowing simultaneous measurement of changes in the expression of thousands of genes. In the past few years, this technology has been used to discover gene function, understand biochemical pathways and regulatory mechanisms, classify dis-

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains Tables A–D.

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¹ The abbreviations used are: 5-FC, 5-fluorocytosine; RT, reverse transcriptase; CFU, colony-forming units; DI, difference index; MOPS, 3-(N-morpholino)propanesulfonic acid; 5-FU, 5-fluorouracil; MMS, methylmethane sulfonate; MIC, minimum inhibitory concentration; MAP, mitogen-activated protein.

ease specimens, and discover drug targets (reviewed in Ref. 13). The yeast *Saccharomyces cerevisiae* is an excellent model organism for the study of antifungal action. Its genome has been fully sequenced and well characterized, and with the development of whole-genome microarrays, it is now possible to monitor globally gene expression changes in response to various experimental conditions. The availability of deletion mutant stocks for *S. cerevisiae* also greatly facilitates the validation of novel hypotheses generated from microarray experiments.² In addition, among the pathogenic fungi, *S. cerevisiae* is most closely related to *Candida albicans*, the major opportunistic fungal pathogen (14). In recent years, *S. cerevisiae*, previously considered to be non-pathogenic, has been reported as an emerging fungal pathogen in immunocompromised individuals (15). Thus, *S. cerevisiae* serves as a valuable model system in the study of fungal infections and identification of new drug targets.

Genomic profiling studies have examined the effects of amphotericin B, 5-FC, and various azoles in both *S. cerevisiae* and *C. albicans* (16–19). These studies provide an excellent overview of the genes expected to change their expression in response to treatment with these drugs. However, these studies have used concentrations that may not be optimal for appropriate between-drug comparisons with regard to their effects. Furthermore, we show here that differences in experimental conditions such as growth media, concentration of drug, and period of drug exposure can contribute to gene expression changes. Because the experimental conditions utilized in our study are different from those used in previous studies, we have been able to identify responses that were not previously reported. In addition, the minimal media conditions used in the current study perhaps better mimic the nutrient-limited environment of macrophages, which phagocytose *C. albicans* cells and allow hyphal development in this pathogen (20). In the present study, genome-wide gene expression profiling was used to compare and characterize changes in *S. cerevisiae* gene expression in response to representatives of four major classes of antifungal agents in an effort to identify drug class-specific and mechanism-independent changes in gene expression.

EXPERIMENTAL PROCEDURES

Antifungal Agents—Ketoconazole and 5-fluorocytosine were obtained from Sigma. Amphotericin B was from ICN Biomedicals (Aurora, OH). A commercially available preparation of caspofungin acetate for injection (Candicid®) was obtained from a local pharmacy. Stock solutions of varying concentrations were made in Me₂SO (Sigma).

Yeast Strains and Media—*S. cerevisiae* S288C (*MAT α* , *SUC2*, *mal*, *mel*, *gal2*, *CUP1*, *flo1*, *flo8-1*), obtained from ATCC (Manassas, VA), was used in the microarray experiments. The *upc2-1* mutant strain (SCY1012, *MAT α* , *ade2-1*, *can 1-1*, *trp1-1*, *ura3-1*, *his3-11*, *15*, *leu2-3*, *112*, *upc2-1 lo*) and its isogenic wild type strain (SCY325) were generously provided by Dr. Stephen Sturley (Columbia University, New York). The *upc2 Δ* strain (*MAT α* , *leu2*, *ura3*, *his3*, *lys2*, *upc2 Δ*) and its isogenic wild type strain (BY4742) were obtained from Invitrogen. Synthetic dextrose (SD) medium, containing 0.67% (w/v) yeast nitrogen base without amino acids and 2% (w/v) dextrose, was used to grow the wild type S288C strain. Synthetic complete (SC) medium, consisting of SD medium supplemented with complete supplement mixture (CSM, Qbiogene, Inc., Carlsbad, CA), was used to grow the *upc2-1* mutant strain, the *upc2 Δ* strain, and their respective isogenic wild type controls. In all cases, the medium was buffered with 0.165 M MOPS (Sigma), and the pH was adjusted to 7.0 with NaOH.

IC₅₀ Determinations—For IC₅₀ determinations in small scale cultures, a single colony of *S. cerevisiae* was resuspended in 0.9% sterile saline. The culture was diluted in SD medium after comparison to the 0.5 McFarland standard to afford a final inoculum of $\sim 3 \times 10^6$ CFU/ml. After dilution, 175 μ l of the culture was inoculated into a 96-well flat bottom culture plate (Corning Inc., Corning, NY) containing 25 μ l of test

compounds that were 2-fold serially diluted in 0.9% sterile saline. The starting concentrations used were 10 μ g/ml for amphotericin B and caspofungin, 50 μ g/ml for 5-fluorocytosine, and 100 μ g/ml for ketoconazole. The culture plate was read at 630 nm prior to and after incubation (30 °C for 17 h) using an EL340 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

For determination of IC₅₀ values in large scale cultures, an overnight culture (started from a single colony) in late exponential phase was used to inoculate 100 ml of SD medium to an A₆₀₀ of 0.1. Multiple cultures were started for each drug in order to test 4–5 different drug concentrations. The cultures were incubated at 30 °C in an environmental shaker (200 rpm) and allowed to recover from stationary phase until an A₆₀₀ of 0.2 was reached. Drug was then added at 2–4-fold serial dilutions into each culture. Two rounds of experiments were conducted, with a broad range of drug concentrations tested in the first round and a narrow range tested in duplicates in the second round. The cultures were grown for 17 h at 30 °C, and a final A₆₀₀ was measured using an Ultrospec 2000 spectrophotometer (Amersham Biosciences).

Cell Culture and Drug Exposure for Microarray Experiments—A single colony of *S. cerevisiae* was inoculated into 25 ml of SD medium and grown overnight at 30 °C in an environmental shaker (200 rpm) until late exponential phase. The culture was used to inoculate 100 ml of SD medium to an A₆₀₀ of 0.1. Two independent 100-ml cultures were grown for each drug. All drug exposures were performed on the same day using the same starting culture to minimize experimental variations. The cultures were incubated at 30 °C in an environmental shaker (200 rpm) and allowed to recover from stationary phase until an A₆₀₀ of 0.2 was reached. Drug was then added to each culture at a concentration equivalent to the IC₅₀ value (concentrations used were 0.12 μ g/ml amphotericin B, 0.02 μ g/ml caspofungin, 0.3 μ g/ml 5-fluorocytosine, and 56 μ g/ml ketoconazole). Control cultures were simultaneously treated with 0.25% Me₂SO. The cultures were allowed to grow until an A₆₀₀ of 0.5 was reached (~ 3 h, the doubling time in SD medium was ~ 2 h). Cells were harvested by centrifugation at $500 \times g$ at 20 °C for 5 min in a Sorvall RC5C Plus centrifuge using an SH-3000 rotor. The cells were flash-frozen in liquid nitrogen and stored at -80 °C.

RNA Preparation—Total RNA was isolated using the Qiagen RNeasy® Midi-kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions with the following modifications: frozen *S. cerevisiae* cells were ground into a powder with a mortar and pestle in liquid nitrogen to facilitate cell disruption. The cell powder was resuspended in 4 ml of lysis buffer provided in the kit and homogenized for 2 min at 30-s intervals at 25,000 rpm using a PT3100 Polytron (Brinkman Instruments). The RNA concentration and purity were determined spectrophotometrically by measuring absorbance at 230, 260, 280, and 320 nm. The purity and integrity of the RNA were confirmed by agarose gel electrophoresis.

Microarray Hybridization and Analysis—Microarray hybridization was performed using the Affymetrix GeneChip® Yeast Genome S98 Array using protocols described by Affymetrix, Inc. (Santa Clara, CA). Data were analyzed using Affymetrix® Microarray Suite 5.0 software. Genes were considered to be differentially expressed if (i) expression changed by at least 2-fold in two independent experiments performed with duplicate RNA samples, (ii) the mRNAs were assigned at least one “present” detection call by the Affymetrix software in both experiments, and (iii) the change in gene expression was in the same direction (“increased” or “decreased”) in both experiments. Genes were annotated using the *Saccharomyces* Genome Data Base³ and Yeast Proteome Data Base (Incyte Corp., Palo Alto, CA). Drug-specific responses were identified by calculating the difference index (DI) described by Nau *et al.* (22) as follows: $DI = \log_2(\text{fold change of gene X in drug exposure A}) - \log_2(\text{average fold change of gene X in all four drug exposures})$.

Quantitative Real Time RT-PCR Assays—An aliquot of the RNA preparations from untreated and treated samples, used in the microarray experiments, was saved for quantitative real time RT-PCR follow-up studies. The RNA samples were treated with DNase I “on column” as per manufacturer's instructions using Qiagen RNeasy® (Qiagen, Inc., Valencia, CA) columns to remove residual DNA contamination. First strand cDNAs were synthesized from 2 μ g of total RNA in a 100- μ l reaction volume using the TaqMan Reverse Transcription

² *Saccharomyces* Genome Deletion Project Home Page (www.sequence.stanford.edu/group/yeast_deletion_project) (accessed June, 2003).

³ K. Dolinski, R. Balakrishnan, K. R. Christie, M. C. Costanzo, S. S. Dwight, S. R. Engel, D. G. Fisk, J. E. Hirschman, E. L. Hong, L. Issel-Tarver, A. Sethuraman, C. L. Theesfeld, G. Binkley, C. Lane, M. Schroeder, S. Dong, S. Weng, R. Andrada, D. Botstein, and J. M. Cherry, *Saccharomyces* Genome Data Base (www.yeastgenome.org) (accessed June, 2003).

TABLE I
Gene-specific primers used for real time RT-PCR assays

Gene	ORF	Primer pairs	Position ^a	Amplicon size
				bp
<i>ERG11</i>	YHR007C	F, 5'-AATGCTCACCAATTCAACATTTCAC-3'	1264	50
		R, 5'-AGAGGAGGCAGAATCTTTGTTCC-3'	1314	
<i>ERG26</i>	YGL001C	F, 5'-TGGACTTATGCTGGAAATGTTGC-3'	646	51
		R, 5'-GTTTCTGTGCAGCTAACACATGC-3'	697	
<i>UPC2</i>	YDR213W	F, 5'-TTCGAGGACTGAAACTGGAAGT-3'	1983	50
		R, 5'-TCTAGGCGGTGAGATGAAACG-3'	2033	
<i>DAN1</i>	YJR150C	F, 5'-GTCTCTCCAAAGCTCAATCTACAG-3'	751	50
		R, 5'-AGATGCTGACGATGTGACAGAAG-3'	801	
<i>AUS1</i>	YOR011W	F, 5'-TTTGCTGGTTACTACTTAACCTACGTGG-3'	4081	49
		R, 5'-AAGACCTTTGGCCATATTCTTGC-3'	4130	
<i>GIT1</i>	YCR098C	F, 5'-ATGCTGGACAGGTGATATGC-3'	1133	53
		R, 5'-CGGTTGCTGACGCTTCACTAC-3'	1186	
<i>TIS11</i>	YLR136C	F, 5'-TCAAACATGGCGACGACAAC-3'	689	50
		R, 5'-AAGTACCAGCTTTACGTAACAGC-3'	739	
<i>HXT5</i>	YHR096C	F, 5'-GAGTCGTATCCCTTAAGAGTACGTGG-3'	1423	49
		R, 5'-GCACCTGCAATCGACATTGC-3'	1472	
<i>YPS3</i>	YLR121C	F, 5'-GCTGTAGATCACAGCAAGTACGAG-3'	688	50
		R, 5'-AACCAACGGGATAGTGTACAGTTG-3'	738	
<i>PHM8</i>	YER037W	F, 5'-AGTGGGTGTGTCGAGCTTTGC-3'	649	50
		R, 5'-GCTTTTCGTTGTATCAATAAACACG-3'	699	
<i>GSC2</i>	YGR032W	F, 5'-TCCATAACTTGGTTCAACCAAGG-3'	5567	47
		R, 5'-ACCCTGTATCATTGTTAGATACGTTC-3'	5614	
<i>MTL1</i>	YGR023W	F, 5'-TCAGAAATTTGGTGATATCGATCC-3'	1317	50
		R, 5'-GGGTTGTCTATTGTTAAGTATATCTTCTGG-3'	1367	
<i>BAG7</i>	YOR134W	F, 5'-TGCTTCCAAGGTCACGTGC-3'	908	50
		R, 5'-GATGTATGGTAAATTTGTTGGAGTCAG-3'	958	
<i>SLT2</i>	YHR030C	F, 5'-TGGAGATGAGACCTGCCACTG-3'	1340	50
		R, 5'-CATTCTGAGGCGGAATATCTGC-3'	1390	
<i>RLM1</i>	YPL089C	F, 5'-AAATACCGGGCTGACTCCATAC-3'	1923	50
		R, 5'-GTGCCTAGTGGTGGTTTGAGCAG-3'	1973	
<i>RNR3</i>	YIL066C	F, 5'-GAAAGCTCAGATAATGAGAAGGATGC-3'	2389	50
		R, 5'-TGATTGTTCGGATGGAAGTGG-3'	2439	
<i>RFA2</i>	YNL312W	F, 5'-TGGAATGATGAAACAACCTTTGG-3'	534	51
		R, 5'-TGATTGCCCATTTGTTGG-3'	585	
<i>HO</i>	YDL227C	F, 5'-ATTACGCCCGAAGGTTTGC-3'	1636	50
		R, 5'-CGCCACATTTTATACACTCTGGTC-3'	1687	
<i>DSE4</i>	YNR067C	F, 5'-TGACCAGTCAATGGAATTGAGG-3'	2886	51
		R, 5'-ATCCTTCATTATACTAATCATCAAGTCACC-3'	2937	
<i>PCL1</i>	YNL289W	F, 5'-CCGATTTAAGTTCTGCTACTCTAGTCG-3'	656	50
		R, 5'-GCCTTCTTAGATCATTACAAGTGTGC-3'	706	

^a Position of 5'-most primer nucleotide in the coding sequence of the gene.

Reagents Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions. Quantitative real time PCRs were performed in triplicate using the GenAmp® 5700 Sequence Detection System (Applied Biosystems). Independent PCRs were performed using the same cDNA for both the gene of interest and 18 S rRNA, using the SYBR® Green PCR Master Mix (Applied Biosystems). Gene-specific primers were designed for the gene of interest and 18 S rRNA using Primer Express® software (Applied Biosystems) and the Amplify program (23). All related coding sequences within the *S. cerevisiae* genome were identified via BLASTN queries of the complete coding sequences using the BLAST server at the *Saccharomyces* Genome Data Base.³ All sequences thus identified were then aligned using the ClustalW alignment function of MegAlign software (DNASTar, Inc., Madison, WI). Gene-specific primer pairs were then manually selected such that at least one primer per pair contained a minimum of two consecutive mismatches at the 3' end when compared against all related *S. cerevisiae* sequences. For almost all genes analyzed, both primers within a pair fulfilled the above criteria and contained numerous additional mismatches when compared against related sequences. The sequences of the primer pairs are listed in Table I. The PCR conditions consisted of denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified using software provided with the GenAmp® 5700 sequence detection system. A negative control reaction in the absence of template (no template control) was also routinely performed in triplicate for each primer pair. The change in fluorescence of SYBR® Green I dye in every cycle was monitored by the GenAmp® 5700 system software, and the threshold cycle (*C_T*) above background for each reaction was calculated. The *C_T* value of 18 S rRNA was subtracted from that of the gene of interest to obtain a ΔC_T value. The

C_T value of an arbitrary calibrator (e.g. untreated sample in the case of up-regulated genes) was subtracted from the ΔC_T value to obtain a $\Delta\Delta C_T$ value. The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta C_T}$.

Susceptibility Testing of *upc2* Mutant Strains—The *upc2Δ* strain was confirmed as recommended by the *Saccharomyces* Genome Deletion Project protocols.² Single colony PCR was performed using the following primers: UPC2-A (5'-TATTGTCTTCAGTGAATGTGCTTGT-3'), UPC2-B (5'-TTCCCTTCATCACACTTAACCTCTTC-3'), UPC2-C (5'-AGGTC-AAGGATAAAGTCTGGTTTCT-3'), UPC2-D (5'-AATTGCCTCTCTATCAAAGTTTCCT-3'), KanB (5'-CTGCAGCGAGGAGCCGTAAT-3'), and KanC (5'-TGATTTTGATGACGAGCGTAAT-3'). The UPC2-A and UPC2-D primers are located upstream and downstream of the *UPC2* coding region, respectively, whereas the UPC2-B and UPC2-C primers are located within the coding region. The KanB and KanC primers are located in the *Kan* gene used to replace *UPC2* in the deletion strain. PCR products were obtained only in the UPC2-A/KanB and UPC2-D/KanC primer combinations, whereas the UPC2-A/UPC2-B and UPC2-C/UPC2-D primer combinations resulted in no PCR products. The *upc2-1* strain (SCY1012) was confirmed by colony PCR with UPC2-C/UPC2-D primers, followed by restriction digest of the amplified PCR product with *Hph*I restriction enzyme. The *upc2-1* mutation (G to A transition at nucleotide 2663) creates a unique *Hph*I restriction site (72). After restriction digestion, the *upc2-1* strain produced two DNA fragments (395 and 80 bp), whereas the corresponding wild type strain (SCY325) produced only one fragment that was 475 bp in size.

For susceptibility testing with ketoconazole, a single colony from a YPD agar plate was grown overnight in SC medium. The culture was diluted in SC medium after comparison to the 0.5 McFarland standard to afford a final inoculum of $\sim 1 \times 10^4$ CFU/ml. After dilution, 180 μ l of the culture was inoculated into a 96-well flat bottom microplate (Corn-

ing Inc., Corning, NY) containing 10 μ l of ergosterol (final concentration of 10 μ g/ml) and 10 μ l of ketoconazole at varying concentrations. The starting concentration of ketoconazole used in the assay was 100 μ g/ml. Ergosterol (Sigma) was added from a stock solution of 0.2 mg/ml prepared in Tween 80/ethanol (1:1 v/v). Ketoconazole was prepared as a stock solution of 10 mg/ml in Me_2SO , which was 3-fold serially diluted in Me_2SO , followed by a 5-fold dilution of each dilution in SC medium. For anaerobic conditions, microplates were incubated in BBL Bio-Bag CO_2 -generating environmental chambers (BD Biosciences). The chambers are supplied with an anaerobic indicator that turns from pink to clear when anaerobiosis has been achieved. The microplates were read at 630 nm, prior to and after incubation (30 °C for 48 h), using an EL340 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

RESULTS AND DISCUSSION

Experimental Design—Because four different classes of drugs were utilized in this study, treatments were restricted to a single time point and a single concentration. Based on previous reports (16–19) in which one doubling time (90 min) was sufficient to detect specific gene expression changes in response to azoles, amphotericin B, and 5-FC, cells were exposed to various drugs for a period of ~ 1.5 times the doubling time in this study. The concentration of drug is critical in these studies because the effect on gene expression may not be detectable if the drug concentration is too low, and secondary drug effects could mask the primary responses if the test concentration is too high. In similar microarray experiments, $0.5\times$ minimum inhibitory concentration (MIC) has been utilized (16–19). However, for some antifungal drugs, this value is sometimes very close to the MIC and causes as much as 80% inhibition (Fig. 1, B–D). For this reason, the IC_{50} concentration was used in this study. The IC_{50} value was determined not only in conventional microplate assays but also in large scale cultures designed to mimic the conditions of the microarray experiments as closely as possible. A minimal medium such as synthetic dextrose medium was used because nutrient-rich media conditions may compensate for the inhibitory effects of the drug and obscure detection of some gene expression responses. “Biological replicates” were included in the experimental design, i.e. two independent experiments were conducted simultaneously, such that two independent cell cultures and drug treatments were initiated, two RNA samples were isolated, and two hybridizations were performed for each drug.

IC_{50} Determinations—Antifungal drug susceptibility assays routinely performed according to the National Committee for Clinical Laboratory Standards (25) require inocula at low cell densities ($\sim 5 \times 10^3$ CFU/ml). Because the aim of this study was to perform microarray analysis, which requires RNA isolations from a large number of cells, it was necessary to modify the currently available protocols. For this reason, inhibition assays were performed with inocula at high cell densities ($\sim 3 \times 10^6$ cells per ml, A_{600} of ~ 0.1) in SD medium. Assays were first performed in microplates as described under “Experimental Procedures.” Absorbance readings were measured 17 h after the experiment was started in order to ensure that cells were in late exponential phase.

The inhibition assays were repeated in large scale experiments because 100-ml cultures would be used in the microarray experiments, and it is possible that IC_{50} values determined in microplate assays would be different under large scale conditions. The results from the microplate and large scale assays are shown in Fig. 1, A–D. Based on these experiments, the IC_{50} values for the four drugs were determined to be the following: 56 ± 8.18 μ g/ml for ketoconazole, 0.14 ± 0.04 μ g/ml for amphotericin B, 0.03 ± 0.01 μ g/ml for caspofungin, and 0.30 ± 0.16 μ g/ml for 5-fluorocytosine. In general, the microplate and large scale assay results were in agreement with each other (Fig. 1E) except for 5-fluorocytosine, which showed 2–4 times

greater IC_{50} values in large scale conditions. The high IC_{50} value observed for ketoconazole is consistent with the fact that ketoconazole is a fungistatic agent. This value is also in agreement with a previous study in which it was reported that increasing the starting inoculum from 1×10^3 to 1×10^7 CFU/ml caused a dramatic reduction in fluconazole activity (26).

Gene Expression Responses to Ketoconazole—A total of 51 genes was found to be responsive to ketoconazole treatment under the experimental conditions used. Of these, 44 genes showed a significant increase in expression, and 7 genes showed a significant decrease in expression. The number of responsive genes in this study is less than the number observed in previous studies (16, 17) because two independent biological replicate experiments were conducted in this study, and only genes that responded similarly in both experiments were selected as responsive genes. In studies by Lee *et al.* (27), it was shown that the number of false positives can be quite high in a microarray study conducted with samples from only one experiment. For example, in a single experiment, 1.8% of the total genes showed a 2-fold change in expression and of these about 45% were false positives, whereas in a duplicate experiment, 0.6% of the total genes showed a 2-fold change in expression and of these only 1% were false positive (27, 28).

The distribution of ketoconazole-responsive genes and their biological roles are shown in Fig. 2. The category of genes with the largest number of responses is the lipid, fatty acid, and sterol metabolism group. As shown in two previous studies, genes involved in ergosterol biosynthesis were up-regulated in response to azole treatment (16, 17). The genes found to be responsive in this study include *ERG1*, *ERG2*, *ERG3*, *ERG4*, *ERG5*, *ERG6*, *ERG11*, *ERG24*, *ERG25*, *ERG26*, and *ERG28*. Because the enzyme encoded by *ERG11* is the direct target of azole antifungals and because its expression is increased in some azole-resistant *C. albicans* strains, it is not surprising that *ERG11* would be induced in response to ketoconazole treatment (29, 30). Except for *ERG1*, most of the responsive genes in this study are functional downstream of *ERG11* (Fig. 3), suggesting that their induction is in response to ergosterol depletion. Comparison of these results with those obtained in previous studies (16, 17) indicates a few discrepancies that could be attributed to differences in strains used, media conditions used (e.g. *ERG1* and *ERG26* not detected in the study by Bammert and Fostel (16) where rich medium was used), time of drug exposure (*ERG10* induced in the study by De Backer *et al.* (17) where cells were treated with itraconazole for 24 h), absence of genes from previous microarrays (e.g. *ERG24* not measured in the De Backer *et al.* study), and previous lack of annotations for some genes (e.g. *ERG28*).

In addition to the ergosterol biosynthesis genes, six additional genes involved in lipid metabolism were also affected in this study. These include *CYB5*, *HES1*, *YSR3*, *INO1*, *ELO1*, and *UPC2* (Fig. 2). The gene *CYB5* encodes cytochrome *b*₅ and is required for suppressing azole hypersensitivity in a *S. cerevisiae* *erg11* mutant (31). The gene *HES1* shows homology to the human oxysterol-binding protein and plays a role in ergosterol biosynthesis, as implied by reduced membrane ergosterol levels in a *hes1* mutant of *S. cerevisiae* (32). The genes *YSR3* (encoding dihydrosphingosine-1-phosphate phosphatase), *INO1* (encoding inositol-1-phosphate synthase), and *ELO1* (encoding a fatty acid elongating enzyme) have been shown to play a role in sphingolipid biosynthesis in *S. cerevisiae* (33–35). It has been shown recently (36) that a mutation in the *ERG26* gene produced defects in sphingolipid metabolism, indicating that sphingolipid metabolism is regulated by sterol levels in *S. cerevisiae* cells. Thus, the effect of ketoconazole on *CYB5*,

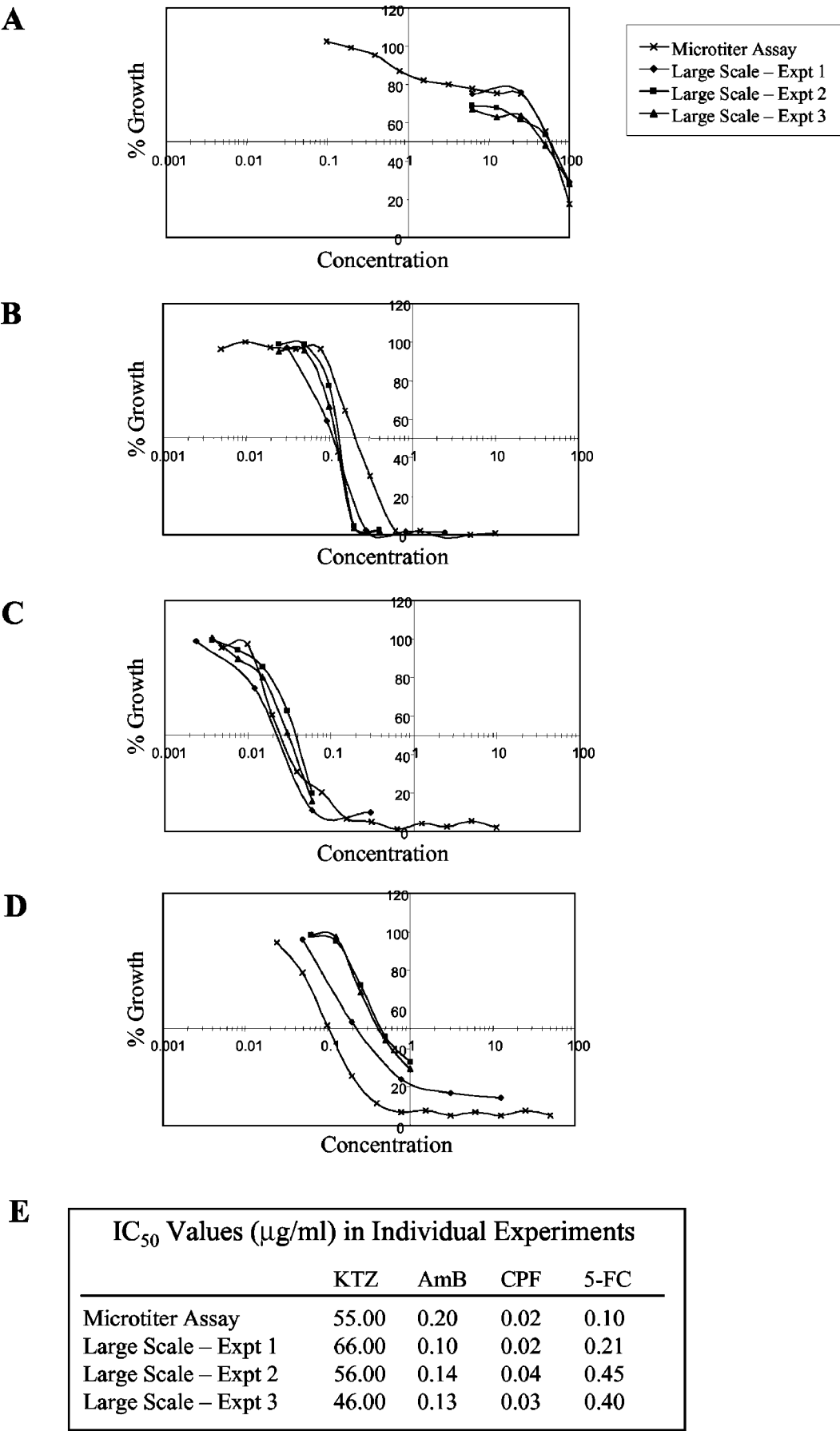
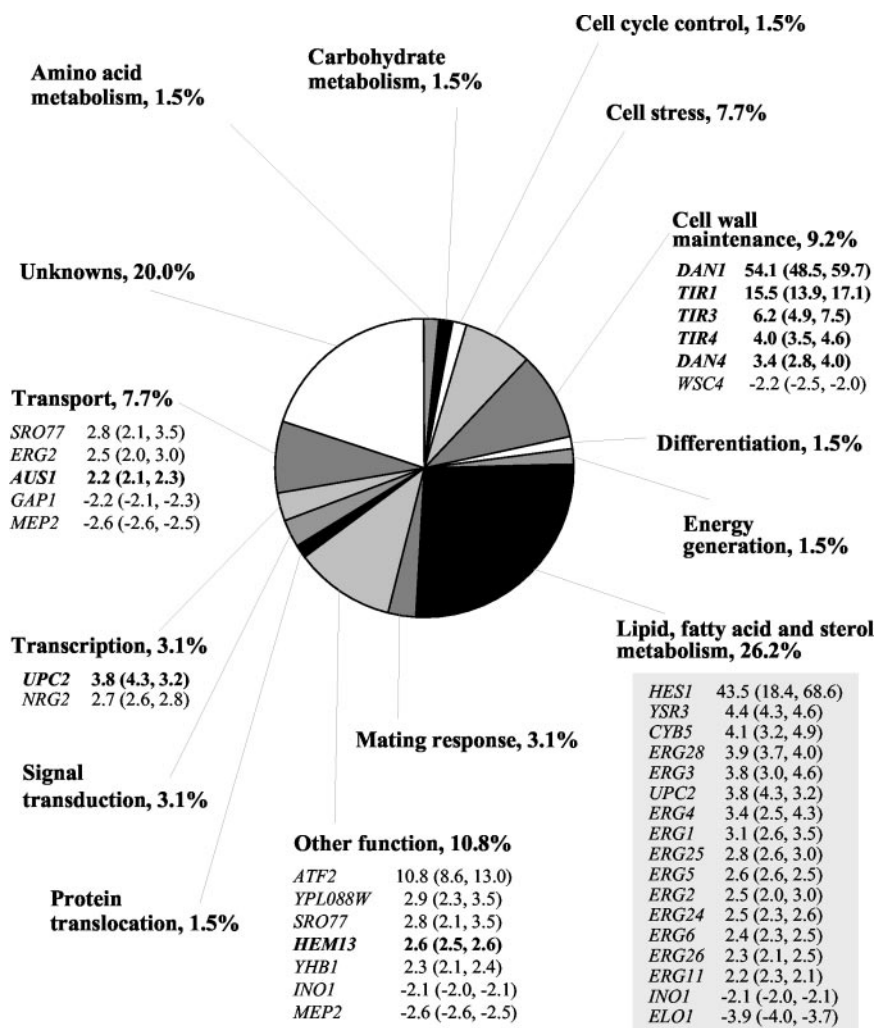


FIG. 1. Determination of IC₅₀ values for ketoconazole (A), amphotericin B (B), caspofungin (C), and 5-fluorocytosine (D). Results are shown from a microtiter assay and three independent assays performed in large scale cultures. E, IC₅₀ values obtained in the individual assays. KTZ, ketoconazole; AmB, amphotericin B; CPF, caspofungin.

FIG. 2. Distribution of ketoconazole-responsive genes. Genes were annotated and assigned to various functional categories using the *Saccharomyces* Genome Data Base and Yeast Proteome Data Base. The average expression ratio from two independent experiments is shown for genes associated with the mechanism of action. In parentheses are the expression ratios obtained in each experiment. Positive numbers indicate induction, and negative numbers indicate repression. Genes in the gray box are indicative of major drug-specific responses. Genes in boldface are summarized in Fig. 3. An annotated list of all the genes in each functional category, including expression ratios from individual experiments, can be found in Supplemental Material Table A.



HES1, *YSR3*, *INO1*, and *ELO1* is in agreement with their roles in sterol or sphingolipid metabolism.

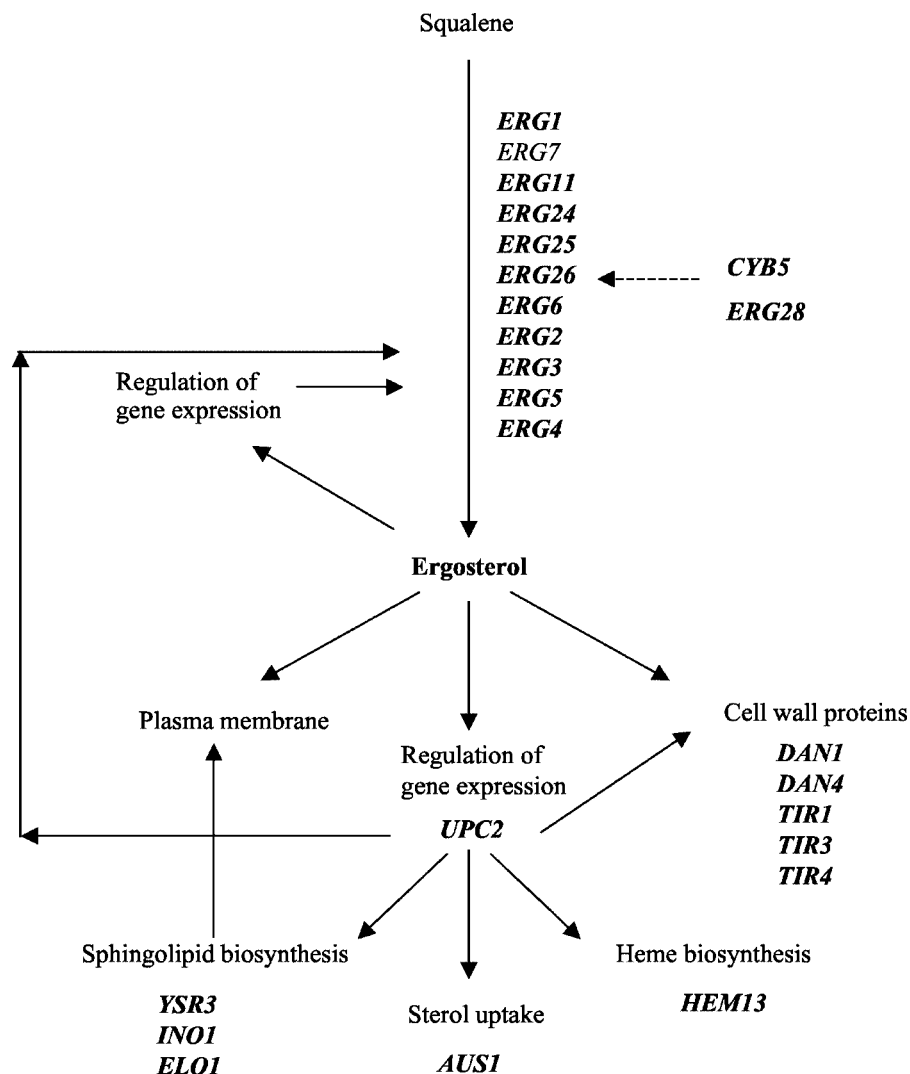
It was interesting to observe that the gene *UPC2* was induced in this study (Fig. 2, *transcription category*). The gene *UPC2* encodes a binucleate zinc cluster transcription factor, which is involved in the regulation of sterol biosynthesis and uptake, and sphingolipid biosynthesis (37, 38). Upc2p is a sterol regulatory element-binding protein, which regulates the transcription of ergosterol biosynthetic genes *ERG2* and *ERG3* (37). Therefore, the induction of *UPC2* in response to ketoconazole is consistent with its role in regulating ergosterol biosynthesis. In addition, *UPC2* is involved in regulating sterol uptake as indicated by the ability of the *upc2-1* mutant to accumulate exogenous sterol in *S. cerevisiae* cells (38). Comparison of normal and *upc2-1* mutant strains by microarray analysis revealed that the genes *UPC2*, *AUS1*, *PDR11*, and *DAN1* are up-regulated in the mutant strain (39). *AUS1* and *PDR11* encode ATP-binding cassette transporters, and *DAN1* codes for a cell wall mannoprotein. Deletions in *AUS1*, *PDR11*, and *DAN1* in the *upc2-1* strain resulted in a decrease in sterol accumulation, suggesting that these genes are required for sterol uptake when sterol biosynthesis is compromised (39).

Most of the genes that were induced in the *upc2-1* mutant strain (39) were also induced in this study in response to ketoconazole (Fig. 2 and Supplemental Material, Table A). These include *HES1*, *CYB5*, *UPC2*, *ERG25*, *ERG24*, *ERG26*, and *ERG11* (lipid metabolism); *DAN1*, *DAN4*, *TIR1*, *TIR3*, and *TIR4* (cell wall maintenance); *AUS1* and *SRO77* (transport);

ATF2 and *HEM13* (other metabolism, heme biosynthesis); *AMS1* (carbohydrate metabolism); *SCM4* (cell cycle control); and *YPL272C*, *YMR317W*, and *YGR131W* (unknowns). All of these genes, except *CYB5*, *YMR317W*, and *YGR131W* have a sterol regulatory element motif in their promoter regions, which has been identified as a Upc2p-binding site. Also, the level of induction of the highly induced genes is very similar between the two studies. For example, *DAN1* was induced ~55-fold in the *upc2-1* mutant, and it was induced ~54-fold in the current study. The induction of *HEM13*, whose product is involved in heme biosynthesis, is consistent with a previous azole microarray result (16). There are several lines of evidence which show that heme plays an important role in sterol synthesis and regulates the transcription of several genes involved in this process (40). It is interesting to note that heme deficiency has been associated with increased sterol uptake in *S. cerevisiae* cells (41).

The similarities between the gene expression responses in the *upc2-1* mutant strain and the ketoconazole-treated cells in the current study support the hypothesis that when sterol biosynthesis is inhibited, cells respond by inducing genes required for sterol uptake from the external environment. It has also been proposed that resistance to azoles could involve multiple mechanisms including alterations in sterol biosynthesis, target site, uptake, and efflux (42). The induction of *UPC2* and some *UPC2*-regulated genes (e.g. *AUS1*, *DAN1*, *DAN4*, *TIR3*, and *TIR4*) appears to be unique to our study. These genes could have been unresponsive in previous microarray studies due to

FIG. 3. Summary of gene expression responses to ketoconazole. Genes in *boldface* were responsive in this study. The relationship between ergosterol biosynthesis and *UPC2*-mediated gene regulation is summarized.



strain differences and media conditions used. In addition, *AUS1* was not annotated at the time these studies were published, and therefore its drug response was not documented.

Taken together, the results in the current study suggest that when *S. cerevisiae* cells grown in minimal medium are exposed for a short time to a sub-inhibitory dose of ketoconazole, they respond by inducing genes that are not only involved in ergosterol biosynthesis but also genes involved in sterol uptake from the external environment. Fig. 3 summarizes the results from this study and highlights the ketoconazole-responsive genes. It also shows the relationship between ergosterol biosynthesis and gene regulation by *UPC2*.

Gene Expression Responses to Amphotericin B—A total of 265 genes was responsive to amphotericin B treatment under the experimental conditions tested. Of these, 185 genes showed a significant increase in expression, and 80 genes showed a significant decrease in expression. Fewer responsive genes were identified in this study relative to a previous study (18), because only genes that responded similarly in two independent experiments were selected as responsive genes in this study.

The distribution of the amphotericin B-responsive genes and their biological roles are shown in Fig. 4. The category of genes with the largest number of responses was the “unknown” group, indicating that many genes that responded to amphotericin B have no previously characterized cellular role. The second largest number of responses was in the transport group, in agreement with results observed by Zhang *et al.* (18). Reg-

ulation of cellular transport is consistent with the mechanism of action of amphotericin B, which binds with membrane ergosterol to form pores that disrupt the membrane and cause leakage of ions and small molecules from the cell (43). The leakage of ions and nutrients from the cell may be at least partially counterbalanced by the induction of genes involved in membrane transport. Genes associated with membrane transport that were induced in this study include *GIT1*, *BAP2*, *BAP3*, *HXT2*, *HXT4*, *HXT5*, *TPO2*, and *PTR2* (Fig. 4). The most highly differentially expressed gene in this category was *GIT1* which showed an ~33-fold induction in response to amphotericin B. This is in agreement with a recent observation that *GIT1* is required for optimal growth of *S. cerevisiae* cells in the presence of 10 μ M nystatin, another polyene antifungal drug (44). Several genes that encode proteins involved in membrane transport were down-regulated in response to amphotericin B, including *MEP2*, *SAM3*, *CTR1*, *SEO1*, *SUL1*, *OPT1*, *GAP1*, and *MMP1* (Fig. 4). It is possible that the induction of certain transport-related genes or the repression of others is necessary to maintain homeostasis within the cell when membranes are damaged.

The category of genes with the next largest number of responses to amphotericin B treatment was the cell stress group. As would be expected, the leakage of cell contents induces stress-related responses in the cell. The genes listed in the cell stress category in Fig. 4 have been shown to respond to various environmental stresses in *S. cerevisiae* cells (45), *e.g.* *HSP12*,

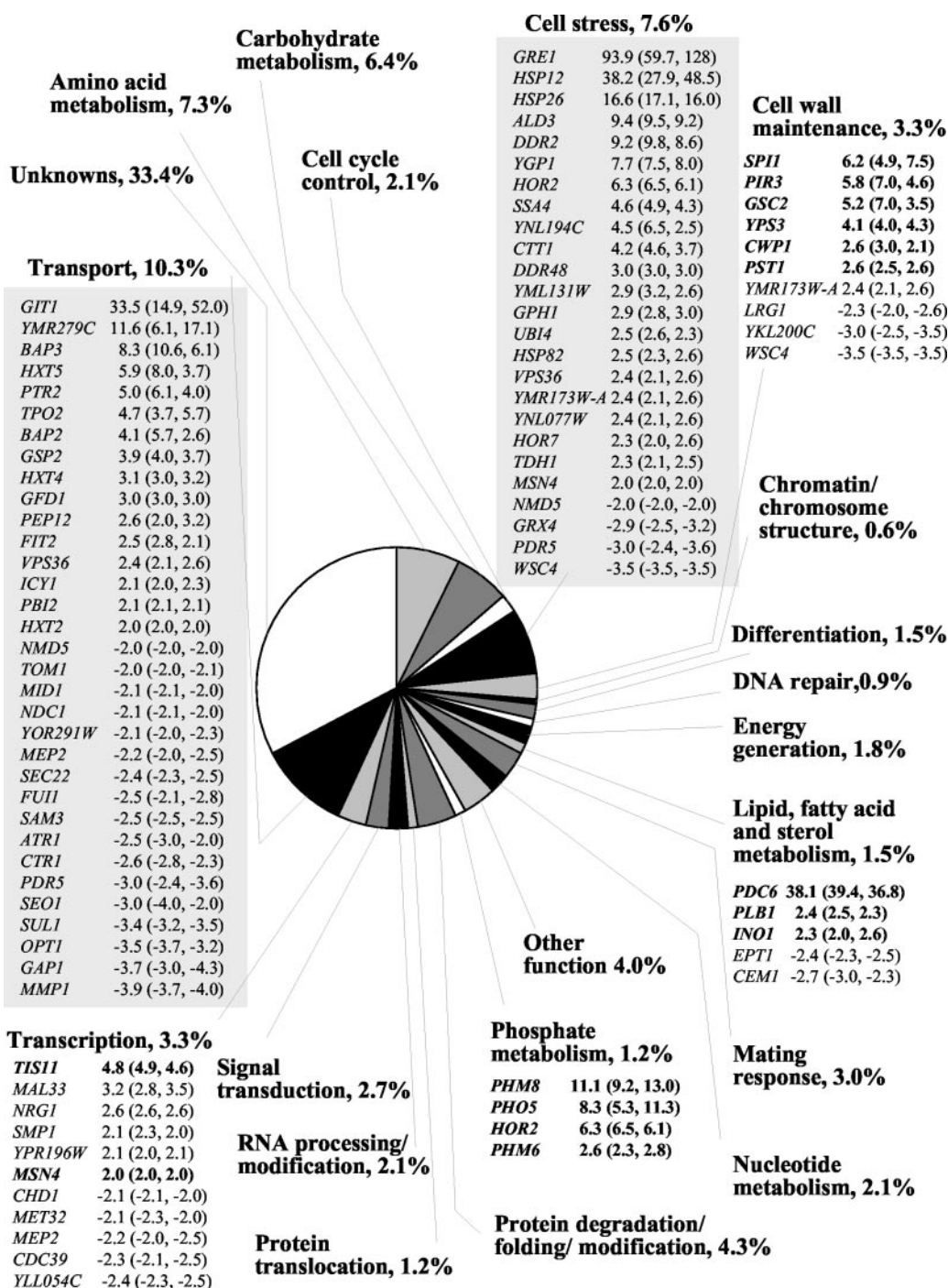


FIG. 4. **Distribution of amphotericin B-responsive genes.** Gene annotations and expression values are as in Fig. 2. Genes in the gray boxes are indicative of major responses to amphotericin B treatment. Genes in boldface are summarized in Fig. 5. An annotated list of all the genes in each functional category, including expression ratios from individual experiments, can be found in Supplemental Material Table B.

HSP26, *HSP82*, and *SSA4* (heat shock, see Ref. 45); *GRE1*, *HOR2*, and *HOR7* (osmotic stress, see Ref. 46), *CTT1* and *DDR48* (oxidative stress, see Ref. 47); *YGP1* (nutrient limitation, see Ref. 48), *ALD3*, *GPH1*, and *TDH1* (ethanol stress, see Ref. 49); and *MSN4*, which is a transcription factor regulating the expression of various stress-response related genes (50). In addition, several genes induced in the environmental stress response (ESR, see Ref. 45) were also induced in response to amphotericin B in this study (Fig. 4 and Supplemental Material, Table B), including *HSP12*, *HSP26*, *UBI4*, *SSE2*, *SSA4*, *SSA1*, *LAP4*, *YPS3*, and *PBI2* (protein folding and degradation); *PDC6*, *TKL2*, *NRG1*, *AMS1*, *HOR2*, *HXK1*, *HXT5*, and *GPH1* (carbohydrate metabolism); *SPI1*, *PIR3*, *GSC2* and

CWP1 (cell wall maintenance); and *GIT1*, *BAP2*, *BAP3*, and *HXT5* (metabolite transport). Several genes repressed in the ESR (45) were also repressed in response to amphotericin B (see Supplemental Material, Table B), including *ADE2*, *FUI1*, and *FUR1* (nucleotide metabolism); and *HAS1* (RNA processing). These results indicate that a general stress response is exerted in *S. cerevisiae* cells in response to amphotericin B treatment.

Reconstructing the cell membrane might be one of the ways in which cells would respond to membrane disruptions caused by amphotericin B. This is reflected in the induction of genes involved in lipid, fatty acid, and sterol metabolism, such as *PLB1* and *INO1* (Fig. 4). In addition, the gene *GIT1* was in-

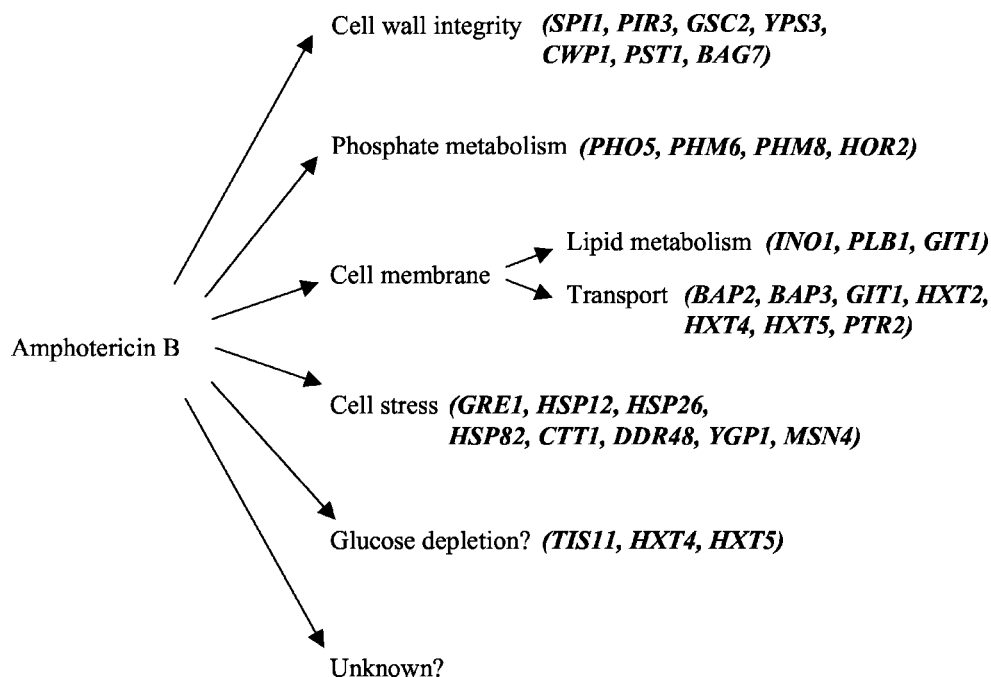


FIG. 5. **Summary of gene expression responses to amphotericin B.** The functional features of the gene expression responses to amphotericin B are summarized.

duced that is involved in the transport of glycerophosphoinositol, a precursor to several phospholipids that are essential membrane components in *S. cerevisiae* cells. The responses involved with phospholipid metabolism are in agreement with the hypothesis that amphotericin B interacts with phospholipids in the cell membrane in order to exert its effect (43).

In addition to genes involved in cell membrane preservation, six genes involved in cell wall maintenance were also induced in response to amphotericin B treatment in this study (Fig. 4), including *SPI1*, *PIR3*, *GSC2*, *YPS3*, *CWP1*, and *PST1*. These results are in agreement with previous observations that cell wall components can affect the interaction of amphotericin B with the cytoplasmic membrane (10). It was shown that stationary phase *C. albicans* cells were more resistant to polyenes than exponential phase cells. It has been suggested that because the turnover of cell wall constituents is slower in stationary phase cells, polyenes would have less access to the cell membrane in that phase. The cell wall maintenance genes that were induced in response to amphotericin B in this study are involved in the cell wall stress-response pathway, which reflects a compensatory reaction to cell wall damage (reviewed in Ref. 51). The gene *BAG7* (a gene in the *signal transduction* category, see Supplemental Material, Table B), which was induced ~56-fold in response to amphotericin B in this study, is also involved in regulating key components of the cell wall stress-response pathway (52).

The correlation between cell wall integrity and polyene binding to cell membrane is also consistent with a recent study conducted on a nearly complete (96% of all annotated genes) collection of gene-deletion mutants in *S. cerevisiae* (44). These mutants were analyzed in parallel, and the fitness contribution of each gene was quantitatively assessed in response to various environmental conditions, including challenge with the antifungal polyene nystatin. Interestingly, two of the deletion strains most sensitive to nystatin, *myo5Δ* and *bro1Δ*, had deletions in genes required for cell wall structure and integrity. This result supports the observation that cell wall components can affect the binding of polyene compounds to the cell membrane. In contrast, the two genes *MYO5* and *BRO1* were not affected by amphotericin B treatment in the current study. As

indicated by Giaever *et al.* (44), there is little correlation between fitness profiling and transcription profiling data. Whether this is due to post-transcriptional regulation of some genes or other factors is not presently understood.

Comparison between the results from this study and the microarray study by Zhang *et al.* (18) revealed several differences in the responses observed, which could be due to differences in the experimental conditions such as media conditions (*e.g.* rich medium was used in the previous study), yeast strains (strain L1190 was used in the previous study), and drug concentration used (*e.g.* 20-fold higher concentration of amphotericin B was used in the previous study). Among the genes that responded similarly between the two studies, the gene *TIS11* is of particular interest (Fig. 4, *transcription* category). *Tis11p* is a glucose-repressible zinc finger protein of unknown function (53). It is possible that glucose depletion in amphotericin B-exposed cells leads to the induction of genes such as *TIS11*, *HXT4*, and *HXT5* (Fig. 4). The induction of *PHO5* in the two microarray studies indicates the need to increase phosphate uptake in response to amphotericin B, phosphate being an essential nutrient required for the synthesis of several cellular components such as lipids, proteins, nucleic acids, and sugars. In addition, the genes *PHM6*, *PHM8*, and *HOR2* that were induced in the current study (Fig. 4) are also regulated by the PHO regulatory system involved in the regulation of phosphate uptake from extracellular sources (54). Thus, membrane reconstruction, cell stress, improvement of cell wall integrity, phosphate uptake, and perhaps glucose depletion appear to be the major responses to amphotericin B (Fig. 5).

Gene Expression Responses to Caspofungin—A total of 192 genes responded to caspofungin treatment under the experimental conditions tested. Of these, 137 genes showed a significant increase in expression, and 55 genes showed a significant decrease in expression. The distribution of the responsive genes and their biological roles are shown in Fig. 6. The category of genes with responses most relevant to the mechanism of caspofungin action, *i.e.* inhibition of β -1,3-glucan synthase, is the cell wall maintenance group. The genes induced in this group include *PIR3*, *CWP1*, *YPS3*, *YLR194C*, *CRH1*, and *PST1* (cell wall-related proteins); *GSC2*, *KRE11*, and *ECM4* (cell wall

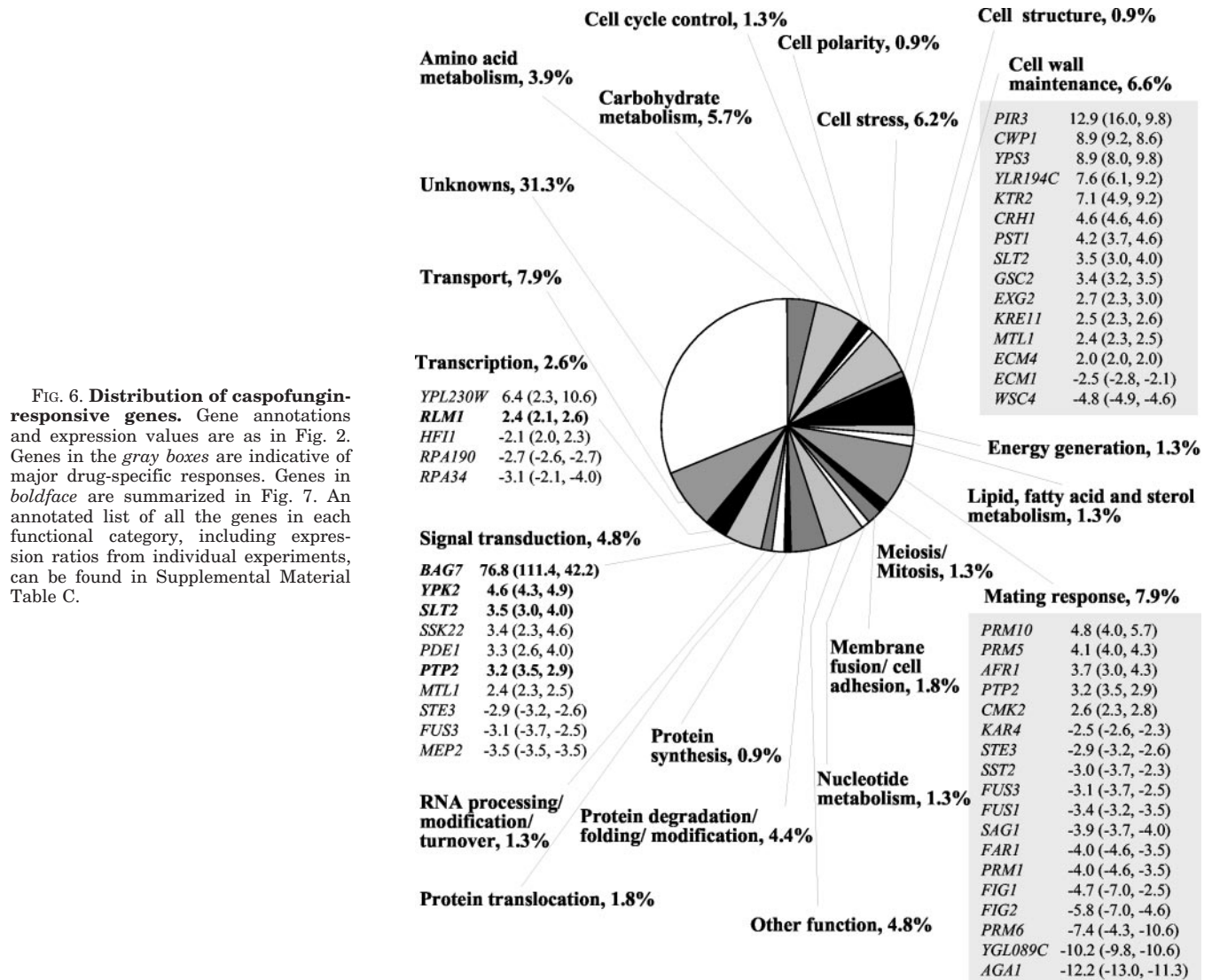


FIG. 6. **Distribution of caspofungin-responsive genes.** Gene annotations and expression values are as in Fig. 2. Genes in the gray boxes are indicative of major drug-specific responses. Genes in boldface are summarized in Fig. 7. An annotated list of all the genes in each functional category, including expression ratios from individual experiments, can be found in Supplemental Material Table C.

biosynthesis); *KTR2* and *EXG2* (cell wall modification); and *SLT2* and *MTL1* (cell wall stress response signal transduction). The genes present in this category belong to the cell wall integrity pathway, which is activated in response to cell wall disrupting agents, such as cell wall lytic enzymes, hypo-osmotic stress, heat stress, cell wall weakening mutations, and chemicals such as the anionic detergent SDS and Calcofluor (an agent that interferes with chitin crystallization) (reviewed in Refs. 51 and 55). These responses include (i) increase in the synthesis of cell wall components, (ii) utilization of alternative mechanisms for incorporation of cell wall proteins, and (iii) increase in cell wall strengthening. The increase in the synthesis of cell wall components in response to cell damage has been shown in several studies utilizing cell wall mutants (reviewed in Ref. 51). A transcription profiling study conducted in a mutant strain of *S. cerevisiae* carrying a disruption in the *FKS1* gene, which encodes the catalytic subunit of β -1,3-glucan synthase enzyme, indicated that several genes encoding glycosylphosphatidylinositol-dependent cell wall proteins and glycosylphosphatidylinositol-attached cell membrane proteins were up-regulated in *fkS1* Δ cells, including *PST1*, *YPS3*, *YLR194C*, *CRH1*, and *CWP1* (56). These genes were also induced in response to caspofungin in the current study (Fig. 6). In addition, the gene *FKS2* (also known as *GSC2*) was induced in the *fkS1* Δ cells. The gene *FKS2* is homologous to *FKS1* and encodes an alternative catalytic subunit of β -1,3-glucan synthases. Thus,

the induction of *GSC2* in the current study is in agreement with the need to synthesize cell wall glucans when glucan biosynthesis is compromised.

Another response to cell wall stress, *i.e.* the increase in cell wall strength, is indicated not only by the induction of genes encoding cell wall proteins in this study but also by the induction of *KRE11*, a gene required for the synthesis of β -1,6-glucan (Fig. 6). The cell wall of *S. cerevisiae* is composed of three major components: glucan, mannoproteins, and chitin (57). Glucan consists of two types of polymers, β -1,3-glucan and β -1,6-glucan that account for 50 and 10% of the dry weight of the cell wall, respectively. Whereas β -1,3-glucan molecules form an internal skeletal layer, β -1,6-glucan plays an important role as a cross-linker connecting the cell wall proteins, chitin and β -1,3-glucan to each other. Thus, when β -1,3-glucan levels are low, as in caspofungin treatment, the cells may compensate by increasing β -1,6-glucan through the induction of *KRE11* and thereby also increasing the strength of the cell wall.

Although several genes involved in cell wall maintenance were induced in response to caspofungin in this study, it is worth noting that the gene *SBE2*, encoding a Golgi protein involved in the transport of cell wall components, was not induced. Overexpression of *SBE2* was shown to confer resistance, whereas deletion of *SBE2* conferred hypersensitivity to caspofungin (58). It is possible that a low level exposure to caspofungin for a short period of time, as was done in

the current study, may not be sufficient to induce the expression of *SBE2*. It is also possible that *SBE2* is not transcriptionally responsive to caspofungin and is regulated posttranscriptionally.

In this study, the induction of the genes *SLT2* (encoding a MAP kinase) and *MTL1* (encoding a transmembrane protein) in response to caspofungin is indicative of the involvement of specific signal transduction pathway components required for cell wall stress-induced responses (Fig. 6, *cell wall maintenance and signal transduction category*). This signal transduction pathway involves a putative sensor in the plasma membrane that senses defects in the cell wall. The gene *MTL1* encodes a membrane protein and has been hypothesized to be a cell wall sensor (reviewed in Ref. 51). Thus, its induction in response to caspofungin is supportive of this proposed role. Downstream of the sensor is a protein designated as Rho1p, a GTPase, that serves as an integral regulatory subunit of the β -1,3-glucan synthase complex. Rho1p can directly activate glucan synthesis through interaction with Fks1p and Fks2p, but it can also activate protein kinase C (encoded by *PKC1*) that in turn can activate a MAP kinase (encoded by *SLT2*) (reviewed in Refs. 51 and 55). Activation of Slk2p leads to the activation of transcription factors that regulate the transcription of several cell wall stress-response genes. A genome-wide survey of *S. cerevisiae* cells in which the cell wall stress-response signaling pathway was strongly active showed that induction of this pathway resulted in the induction of genes such as *SLT2*, *FKS2*, *PST1*, *CRH1*, *CWP1*, and *PIR3* (59). It is interesting to note that one of the transcription factors activated by *SLT2* is Rlm1p. Jung and Levin (59) have also shown that the transcriptional induction, caused by overactivation of the cell wall stress-response signaling pathway, was abolished in an *rlm1Δ* mutant. This result indicates that Rlm1p plays an important role in regulating the expression of cell wall stress-response genes. Interestingly, the gene *RLM1* was induced in response to caspofungin in the current study (Fig. 6, *transcription category*). Thus, the induction of *MTL1*, *SLT2*, and *RLM1* in this study is consistent with their roles in the cell wall integrity pathway.

Another component of the cell wall stress-response pathway is the GTPase-activating protein Bag7p that activates Rho1p *in vitro* (reviewed in Ref. 51). Bag7p also suppresses the lethality of Rho1p hyperactivation in response to cell wall damage (52). It has been suggested that Bag7p interacts with Rho1p and is involved in controlling actin cytoskeleton reorganization. It is worth noting that the gene *BAG7* was induced ~76-fold in response to caspofungin in this study (Fig. 6, *signal transduction category*). The genes *YPK2* and *PTP2* were also induced in the current study (Fig. 6, *signal transduction category*). *YPK2* encodes a yeast protein kinase that acts as a regulator of the cell wall stress response MAP kinase cascade (60). Mutants in *S. cerevisiae* lacking *YPK2* display severely reduced activation of the MAP kinase pathway. The *PTP2* gene encodes a protein-tyrosine phosphatase that can also regulate this kinase pathway by inactivating the MAP kinase encoded by *SLT2* (61). Thus, *YPK2* and *PTP2* regulate the cell wall integrity pathway by controlling the phosphorylation and dephosphorylation of kinases involved in this pathway. The *PTP2* gene was also induced in *fks1Δ* mutants of *S. cerevisiae* (56).

Taken together, these results suggest that exposure to caspofungin, an inhibitor of β -1,3-glucan synthase, causes the cells to improve their cell wall integrity by triggering components of the cell wall stress-response pathway including a cell wall sensor (*MTL1*), a MAP kinase (*SLT2*), regulators of the MAP kinase (*YPK2* and *PTP2*), a GTPase activator (*BAG7*), a transcription factor (*RLM1*), and several genes regulated by Rlm1p

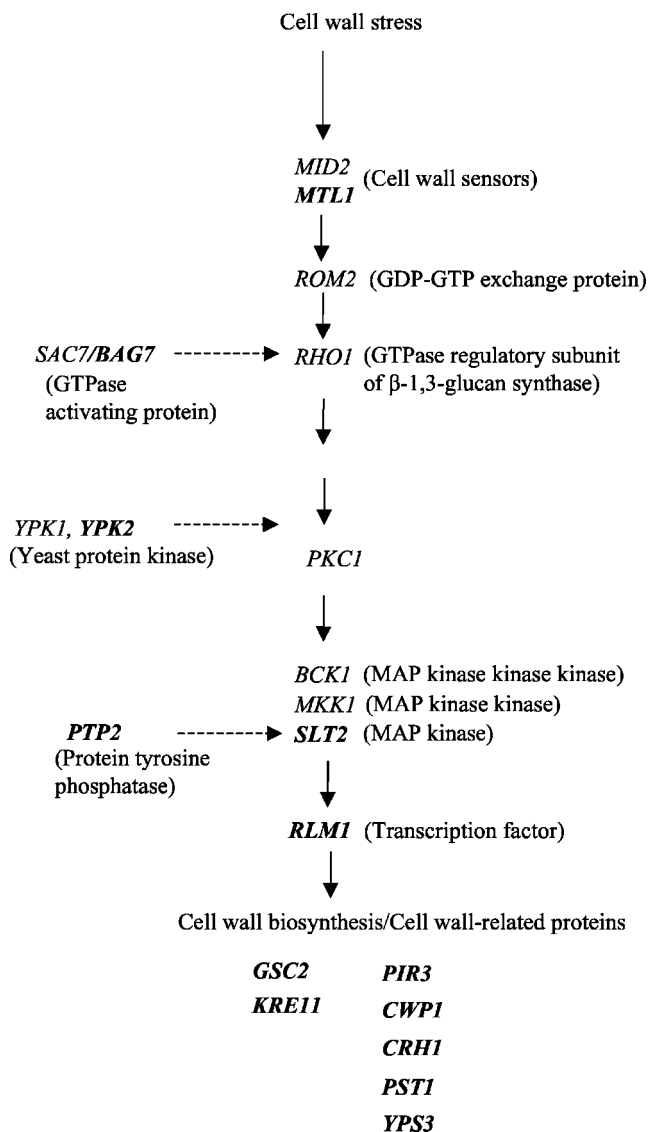


FIG. 7. Summary of gene expression responses to caspofungin. Genes in **boldface** were responsive in this study. Various components of the cell wall stress-response pathway are summarized.

that are either cell wall-related proteins (*PIR3*, *CWP1*, *YPS3*, *YLR194C*, *CRH1*, and *PST1*) or cell wall biosynthesis genes (*GSC2* and *KRE11*). Fig. 7 summarizes the results from this study and highlights the caspofungin-responsive genes and the various components of the cell wall integrity pathway.

Apart from the response in cell wall integrity pathway, caspofungin treatment resulted in the down-regulation of genes involved in mating response (Fig. 6). These include *AGA1*, *FIG1*, *FIG2*, *SAG1*, *FUS1*, *FUS3*, *SST2*, *STE3*, and *KAR4*. The mating response requires the formation of a mating projection, adhesion of cell walls between two cells, and degradation of the cell walls to allow the plasma membranes to fuse (51). This response is in opposition to the cell wall integrity response, which appears to be the major response to caspofungin treatment. Therefore, it is not surprising that mating response genes are down-regulated in response to caspofungin.

Several genes involved in small molecule transport and vesicular transport were either up- or down-regulated in response to caspofungin (Fig. 6). It is possible that damage to the cell wall would cause defects in the plasma membrane affecting transport of small molecules through the membrane. This would be compensated for by the induction of genes involved in facilitating transport.

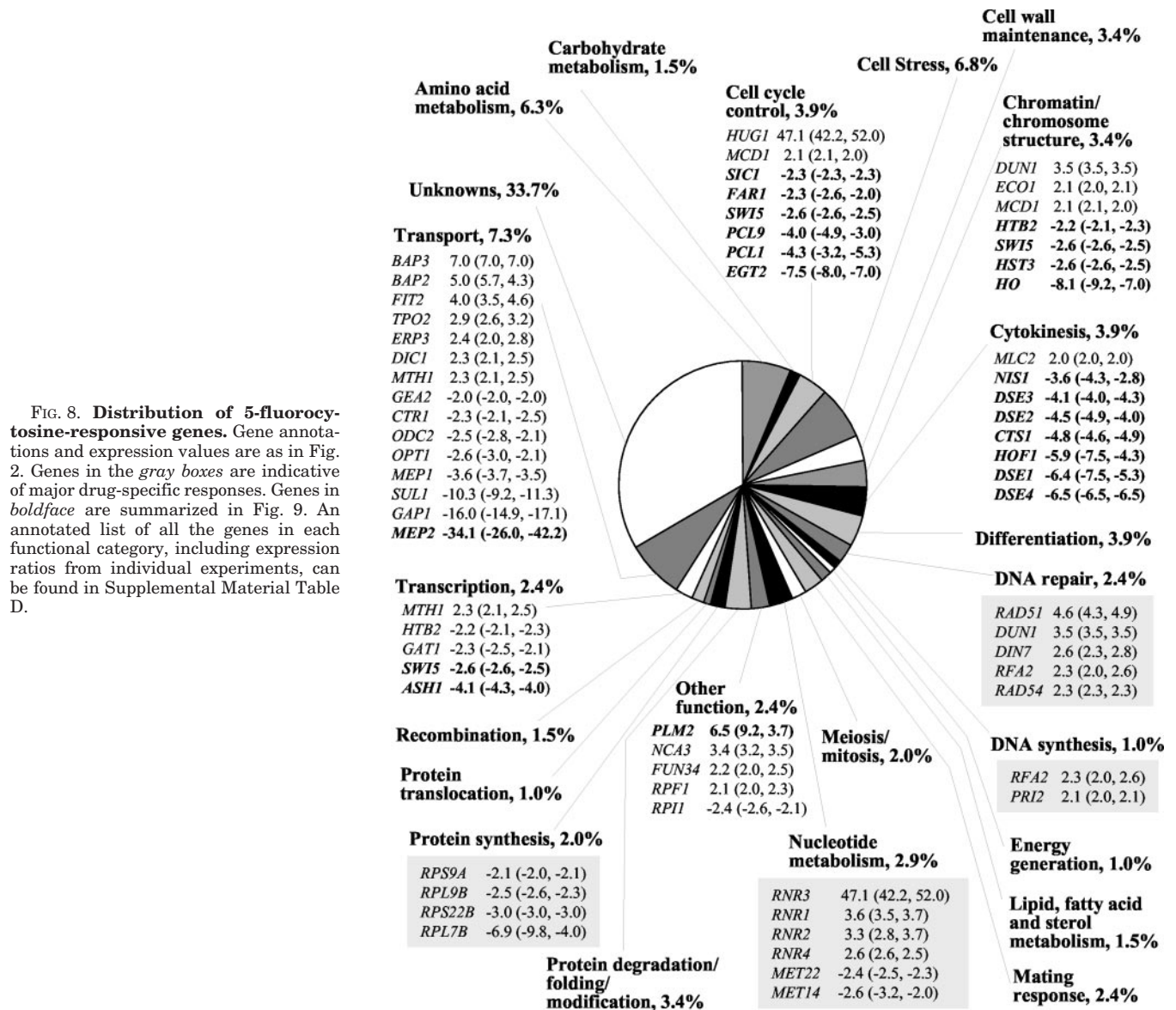


FIG. 8. **Distribution of 5-fluorocytosine-responsive genes.** Gene annotations and expression values are as in Fig. 2. Genes in the gray boxes are indicative of major drug-specific responses. Genes in boldface are summarized in Fig. 9. An annotated list of all the genes in each functional category, including expression ratios from individual experiments, can be found in Supplemental Material Table D.

As observed with amphotericin B, the exposure to caspofungin also triggered a cell stress response and several genes in the cell stress category were induced (Fig. 6 and Supplemental Material, Table C). These include *HSP12* and *UBI4* (heat stress, see Ref. 45); *GRE1* and *HOR2* (osmotic stress, see Ref. 46); *CTT1*, *DDR2*, and *NTH1* (oxidative stress, see Ref. 47); and *ALD3* and *GPH1* (ethanol stress, see Ref. 49). Also several genes induced in the ESR (45) were also induced in response to caspofungin (see Supplemental Material, Table C). These include *HSP12*, *UBI4*, *YPS6*, and *PRB1* (protein folding and degradation); *HXT5*, *AMS1*, *GPH1*, *PFK26*, *ALD4*, and *HXX1* (carbohydrate metabolism); *PIR3*, *CWP1*, and *GSC2* (cell wall maintenance); and *BAP2*, *BAP3*, *HXT5*, and *AGP2* (metabolite transport). Several genes repressed in the ESR (45) were also repressed in response to caspofungin (see Supplemental Material, Table C), including *ADE4*, *FUI1*, and *AAH1* (nucleotide metabolism) and *HAS1*, *MTR4*, and *MAK11* (RNA processing and modification). As with amphotericin B, a large number of genes that responded to caspofungin treatment belong to the “unknowns” category. Perhaps some of these genes play an important role in the cell wall stress-response pathway because several components of this pathway remain unidentified including factors involved in sensing cell wall defects, in reorga-

nizing the cell wall, and in mediating specific responses originating from Rho1p.

Gene Expression Responses to 5-Fluorocytosine—A total of 167 genes responded to treatment with 5-FC under the experimental conditions tested. Of these, 99 genes showed a significant increase in expression, and 68 genes showed a significant decrease in expression. The distribution of the responsive genes and their biological roles are shown in Fig. 8. The responses associated with the mechanism of 5-FC action fell into the following categories: DNA synthesis, protein synthesis, nucleotide metabolism, and DNA repair. These results are in agreement with a previous microarray study in *S. cerevisiae* with 5-FC (19). The drug 5-FC enters fungal cells with the help of a permease enzyme and is converted to 5-fluorouracil (5-FU) by the enzyme cytosine deaminase. Subsequently, 5-FU is converted to 5-fluorouridine phosphate which is incorporated into RNA, resulting in the inhibition of protein synthesis. In addition, 5-FU is also converted to 5-fluorodeoxyuridine monophosphate, a strong inhibitor of thymidylate synthase, an enzyme involved in DNA synthesis and nuclear division. Thus, 5-FC acts by interfering with nucleotide metabolism, as well as RNA, DNA, and protein synthesis in the fungal cell (reviewed in Ref. 10).

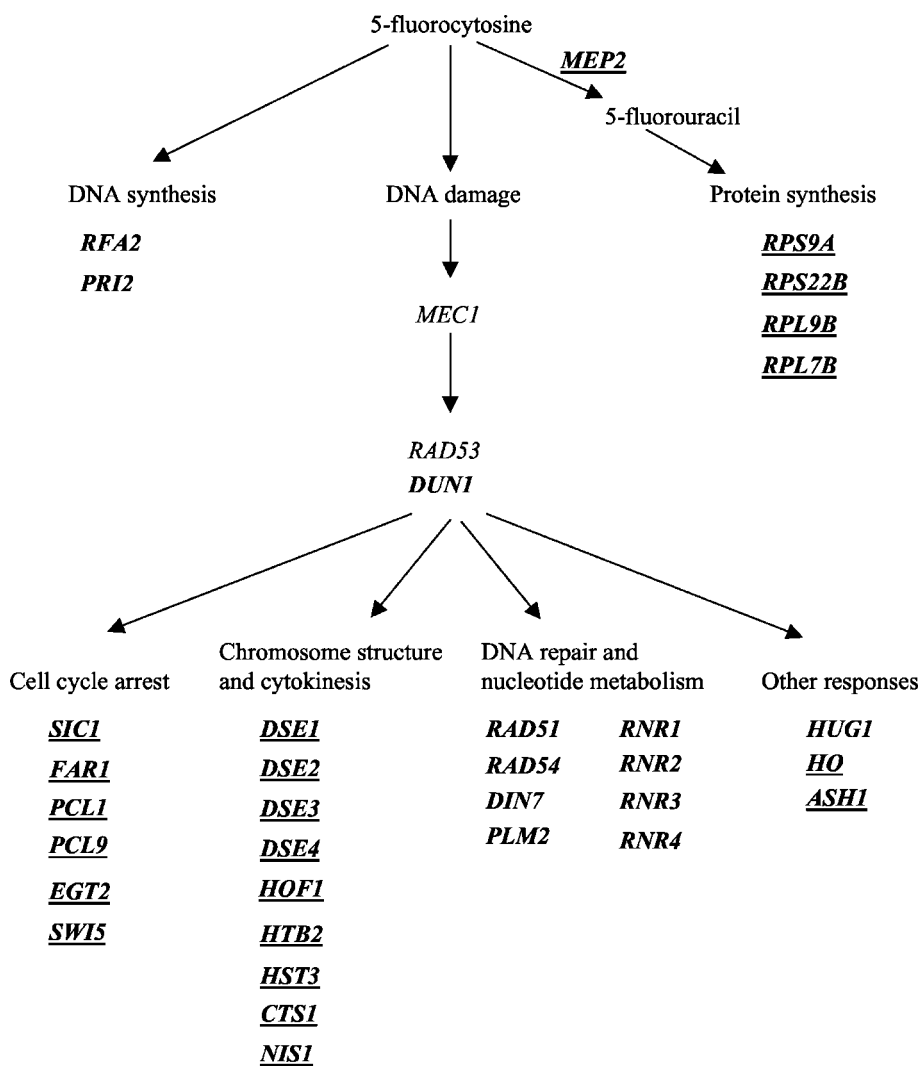


FIG. 9. Summary of gene expression responses to 5-fluorocytosine. Genes in **boldface** were responsive in this study. Underlined genes were repressed by 5-fluorocytosine treatment. Various components of the DNA damage response pathway are summarized.

The effect of 5-FC on protein synthesis was reflected in the repression of genes involved in this process, including genes encoding ribosomal proteins *RPS9A*, *RPL9B*, *RPS22B*, and *RPL7B* (Fig. 8). Similarly, the effect on DNA synthesis was reflected in the induction of genes involved in this process, including *RFA2* (encoding DNA replication factor A) and *PRI2* (encoding DNA primase) (Fig. 8). This result indicates that the cells responded to the inhibition of DNA synthesis by inducing genes required for DNA synthesis. In addition, the cells responded by inducing genes involved in DNA-damage repair. These include the DNA repair genes *RAD51*, *RAD54*, *DUN1*, and *DIN7* and the nucleotide metabolism genes *RNR1*, *RNR2*, *RNR3*, and *RNR4* (Fig. 8). These genes belong to the “DNA damage signature cluster” and are induced in response to DNA-damaging agents such as methylmethane sulfonate (MMS) and ionizing radiation (62). The gene *PLM2* has been implicated in the maintenance of the 2- μ m plasmid in yeast and is also included in this cluster (62). Notably, *PLM2* was also induced in response to 5-FC in this study (represented in the *other function* category, Fig. 8). The genes *RAD51* and *RAD54* are involved in homologous recombination and the repair of double-strand DNA breaks (63). The gene *DUN1* is involved in cell cycle arrest and transcriptional regulation of genes in DNA damage response (64). The gene *DIN7* encodes a protein that is a structural homolog of the DNA repair genes *RAD2* and *RAD27* (65). The *RNR* genes encode subunits of the enzyme ribonucleotide reductase that catalyzes the rate-limit-

ing step of deoxyribonucleotide biosynthesis (reviewed in Ref. 66). Their induction may be required to increase or alter the synthesis of nucleotide pools for DNA replication. The high level of induction of *RNR3* (~47 times) is consistent with previous observations where *RNR3* transcript levels increased up to 100-fold upon DNA damage (67). Thus, the induction of genes in the DNA repair and nucleotide metabolism category is consistent with the need to repair the DNA damage exerted by 5-FC.

In addition to DNA repair, cells also respond to DNA damage by arresting the cell cycle to provide time for repair. The DNA damage-response mechanism generates a signal that arrests cells in the G₁ phase of the cell cycle, slows down S phase (DNA synthesis), and arrests cells in the G₂ phase (reviewed in Ref. 68). Several cell cycle-regulated genes repressed by DNA-damaging agents such as MMS and ionizing radiation (60) were also repressed in response to 5-FC treatment in this study (Fig. 7). These genes include *SIC1*, *FAR1*, *PCL9*, and *EGT2* (cell cycle control); *HTB2* and *HST3* (chromosome/chromatin structure); *CTS1*, *HOF1*, *NIS1*, *DSE1*, *DSE2*, *DSE3*, and *DSE4* (cytokinesis); and *SWI5* and *ASH1* (transcription).

The genes *CTS1* and *EGT2* were also repressed in response to 5-FC in a previous microarray study (19). It was also shown that treatment of *S. cerevisiae* cells with 5-FC inhibited the separation of daughter cells from their mother cells (19), consistent with its effect on the cell cycle and cytokinesis. Although there was general agreement in the types of responses

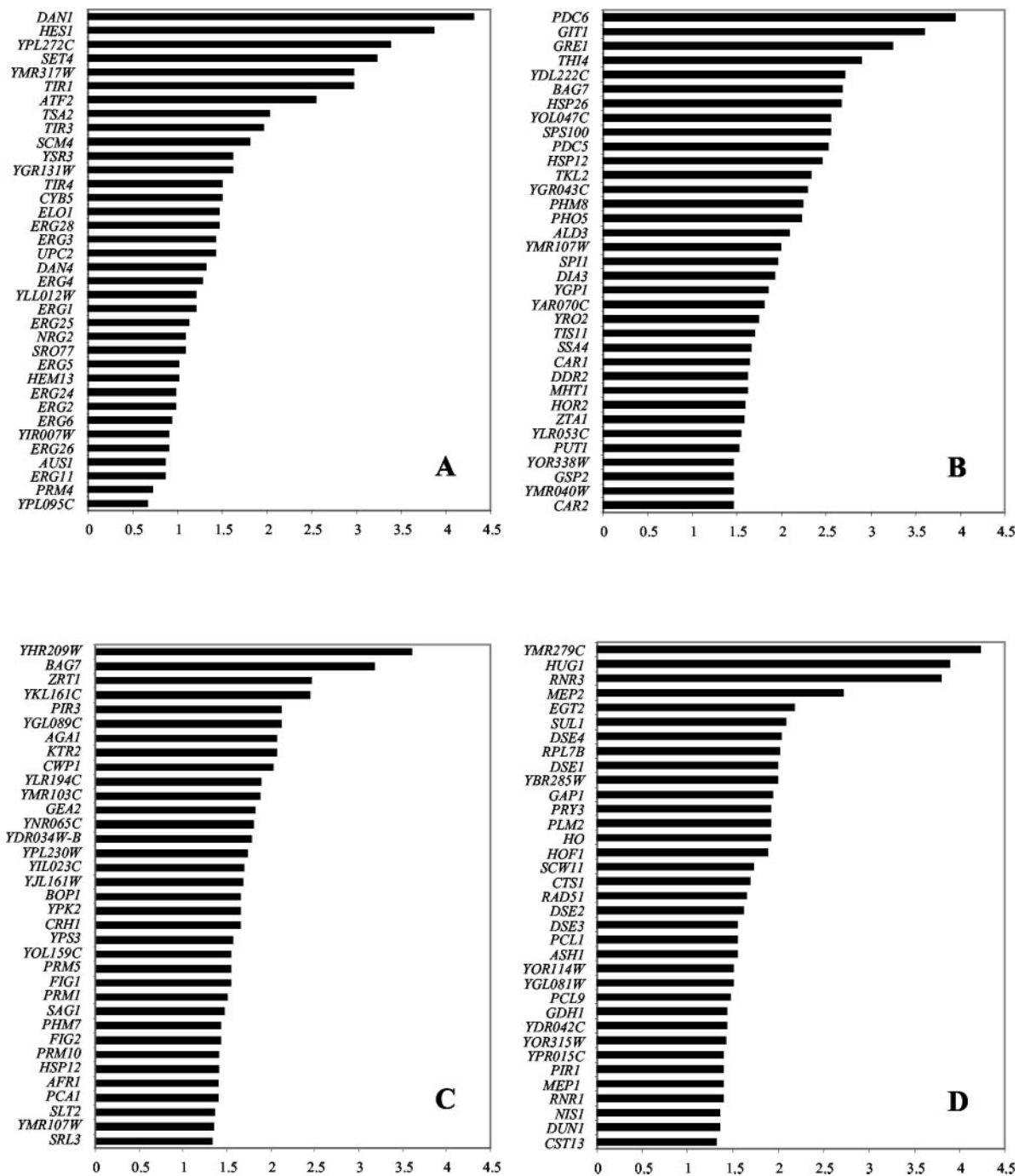


FIG. 10. Drug-specific gene expression responses to ketoconazole (A), amphotericin B (B), caspofungin (C), and 5-fluorocytosine (D). The x axis represents the DI value. The difference index was calculated as follows: $DI = \log_2(\text{fold change of gene } X \text{ in drug exposure A}) - \log_2(\text{average fold change of gene } X \text{ in all drug exposures})$. The DI represents how expression levels induced by one drug differ from the average expression level induced by all four drugs studied.

to 5-FC between the current study and the previous study by Zhang *et al.* (19), only 13 genes responded similarly in the two studies. This could be due to differences in the strains (L1190 was used in the previous study), media (rich medium was used in the previous study), and drug concentration used (~80-fold higher concentration of 5-FC was used in the previous study).

An additional gene that was down-regulated in response to 5-FC treatment in this study was the *HO* gene in the chromatin/chromosome category (Fig. 8). The *HO* gene encodes a homing endonuclease that introduces site-specific double-strand breaks in DNA at the *MAT* locus causing mating-type switching (69). Because 5-FC induces the DNA damage repair pathway, it makes sense that a gene such as *HO* that damages DNA

would be down-regulated. The *HO* gene is regulated not only transcriptionally during late G_1 phase of the cell cycle but is also regulated post-transcriptionally during DNA damage response when the *HO* protein is degraded through the ubiquitin-26 S proteasome system (69). Based on the current study with 5-FC, it appears that *HO* is also regulated at the transcriptional level by the DNA damage-response pathway.

It is interesting to note that most of the cell cycle-regulated genes were down-regulated in response to 5-FC; however, the gene *HUG1* in the cell cycle control category was strongly induced (~47-fold) in this study (Fig. 8). Transcription of *HUG1* is induced in response to DNA damage and replication arrest (induced by hydroxyurea and UV and γ -radiation, see

TABLE II
Responses observed with all four drugs

Cellular role	ORF	Gene	Description
Cell cycle control	YJL157C	<i>FAR1</i> ^a	Factor arrest protein
Cell stress	YFL014W	<i>HSP12</i>	12-kDa heat shock protein
	YOL053C-A	<i>DDR2</i>	DNA damage-responsive
	YPL223C	<i>GRE1</i>	Induced by osmotic stress
Cell wall maintenance	YHL028W	<i>WSC4</i> ^b	Putative integral membrane protein with novel cysteine motif
	YDR055W	<i>PST1</i>	GPI ^c -attached protein
Lipid, fatty acid and sterol metabolism	YJL153C	<i>INO1</i>	L-Myo-inositol-1-phosphate synthase
Mating response	YNR044W	<i>AGA1</i>	Anchorage subunit of α-agglutinin
	YML047C	<i>PRM6</i>	Pheromone-regulated membrane protein
Protein degradation	YLR121C	<i>YPS3</i>	GPI-anchored aspartic protease
Transport	YBR068C	<i>BAP2</i>	Probable amino acid permease for leucine, valine, isoleucine
	YDR046C	<i>BAP3</i>	Valine transporter
	YKR039W	<i>GAP1</i>	General amino acid permease
	YNL142W	<i>MEP2</i>	Ammonium transport protein
	YJL212C	<i>OPT1</i>	Oligopeptide transporter
	YGR138C	<i>TPO2</i>	Polyamine transport protein
Unknown	YLR267W	<i>BOP2</i>	Bypass of PAM1
	YKR091W	<i>SRL3</i>	Suppressor of Rad53 null lethality
	YHR138C		Homologous to PBI2, proteinase inhibitor
	YGR043C		Hypothetical ORF ^d
	YPL088W		Hypothetical ORF
	YBR005W		Hypothetical ORF
	YGR146C		Hypothetical ORF
	YGL121C		Hypothetical ORF

^a Non-highlighted genes responded in at least three of the four drug treatments.

^b Highlighted genes responded in all four drug treatments.

^c GPI, glycosylphosphatidylinositol.

^d ORF, open reading frame.

Ref. 62). The *HUG1* gene has been identified as a component of the DNA-damage checkpoint response using deletion and over-expression mutants of *S. cerevisiae* (70). Thus, the induction of *HUG1* expression in response to 5-FC is consistent with its role in DNA damage response.

In addition to responses related to the mechanism of action of 5-FC, there was also a general induction of stress response (Fig. 8 and Supplemental Material, Table D). Genes induced in the cell stress category included *HSP12* and *HSP26* (heat stress, see Ref. 45); *GRE1* (osmotic stress, see Ref. 46); *DDR2* (oxidative stress, see Ref. 47); and *YGP1*, *XBPI*, *GTT1*, and *SHC1* (various stress conditions including heat, nitrogen depletion, and hydrogen peroxide, see Ref. 45). In addition, several genes induced in the ESR (45) were also induced in response to 5-FC treatment (Fig. 8 and Supplemental Material, Table D), including *HSP12*, *HSP26*, *YRO2*, *SSE2*, and *YPS3* (protein folding and degradation); *MTH1* (carbohydrate metabolism); *ECM4* and *SHC1* (cell wall maintenance); and *BAP2* and *BAP3* (metabolite transport). This result is consistent with previous observations in which the ESR was rapidly induced in response to DNA damage by MMS and ionizing radiation in *S. cerevisiae* cells (62).

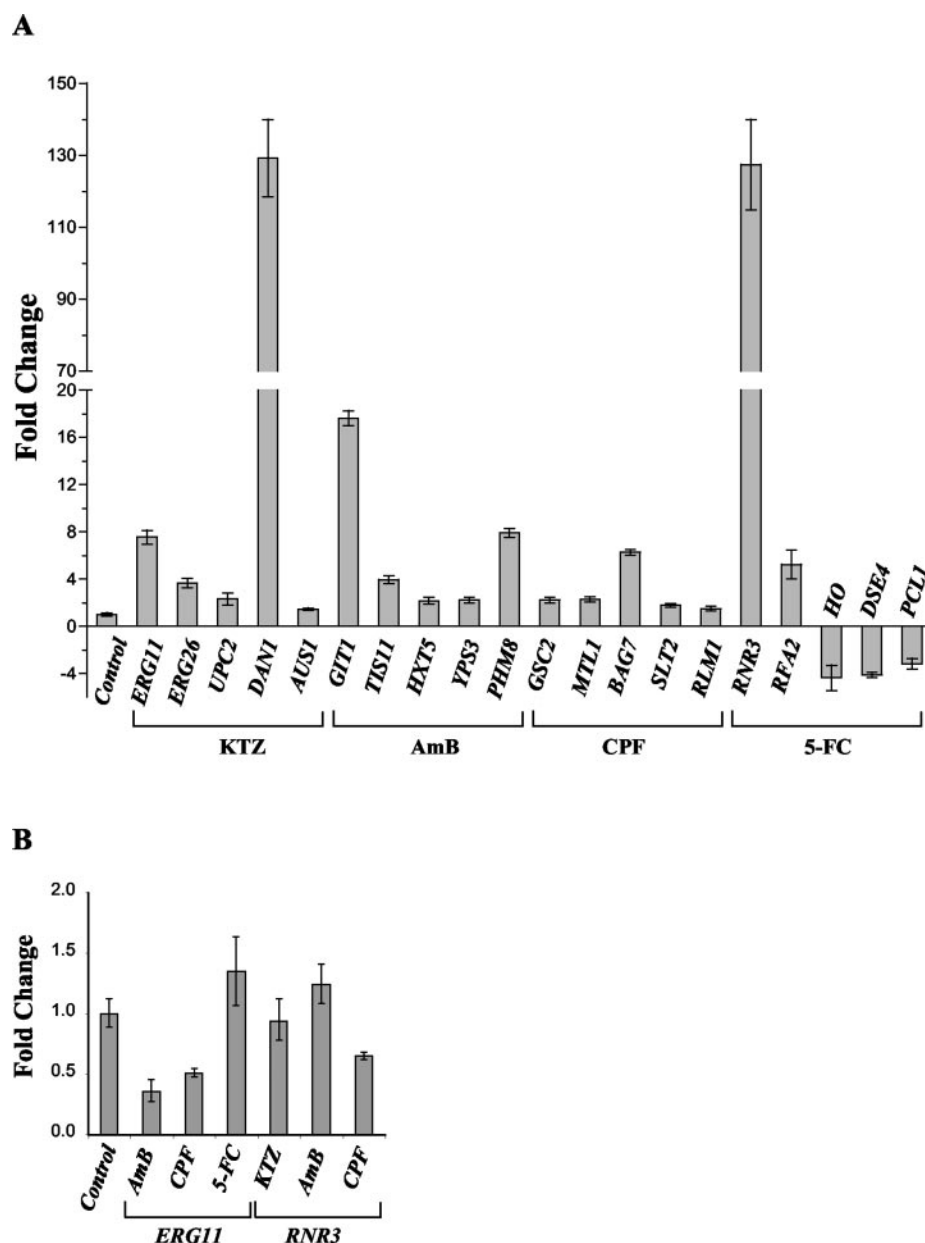
There were also several genes in the transport category that were affected by 5-FC; in particular, the gene *MEP2* was repressed ~34-fold (Fig. 8). The gene *MEP2* encodes an NH₄⁺ transport protein that is down-regulated when *S. cerevisiae* cells are grown in the presence of an appropriate nitrogen source (71). It is possible that the strong repression of *MEP2* in 5-FC-treated cells is due to the build up of NH₄⁺ resulting from the deamination of 5-FC to 5-FU by cytosine deaminase. *MEP2* was also down-regulated in response to the DNA damage-inducing agents, MMS and ionizing radiation (62). As with caspofungin and amphotericin B, there are several genes in the unknown category that respond to 5-FC treatment. Some of these may also play a function in DNA damage response or alternatively, they may participate in an as-yet-unknown re-

sponse to 5-FC. Fig. 9 summarizes the responses to 5-FC and highlights the genes whose expression was affected in this study.

Drug-specific Gene Expression Responses—In order to identify the gene expression responses specific to each drug tested, a difference index (DI) was generated (22). The difference index was calculated as follows, DI = log₂(fold change of gene *X* in drug exposure A) – log₂(average fold change of gene *X* in all four drug exposures). Thus, the DI represents how expression levels induced by one drug differ from the average expression level induced by all four drugs studied. Because the absolute value of fold changes were used as the log argument, genes that had negative expression values (*i.e.* were down-regulated) were assigned positive values. Thirty five genes with the highest DI values were represented in a graph (DI value *versus* gene) for each drug (Fig. 10). These graphs provide a clear indication of drug-specific responses. For example, responses specific to ketoconazole were in ergosterol pathway genes and in Upc2p-regulated genes (Fig. 10A). Specific responses to amphotericin B included genes in the following categories: cell stress, phosphate metabolism, transport, and cell wall integrity (Fig. 10B). Caspofungin-specific responses were in genes represented in the cell wall stress-response pathway and the mating response category (Fig. 10C). Effects specific to 5-fluorocytosine represented genes in the DNA damage repair, nucleotide metabolism, protein synthesis, cytokinesis, and cell cycle control categories (Fig. 10D). In addition, there are several genes in the unknown category that show specific responses to each of the four drugs. This may suggest a possible function of these genes in drug sensitivity or resistance. Thus, the DI graphs make it possible to generate “signature profiles” that represent specific gene expression responses to each of the four drugs tested. These signature profiles will be useful in determining the mechanism of action of novel compounds with antifungal activity.

Responses Observed with All Four Drugs—Table II shows a

FIG. 11. Quantitative real time RT-PCR analysis of genes identified as differentially expressed by microarray experiments. Twenty genes identified as differentially expressed by microarray analysis were examined by quantitative real time RT-PCR with gene-specific primers. Assays were performed in triplicate with SYBR® Green I dye using the GenAmp® 5700 Sequence Detection System. Data were normalized to an internal control (18 S rRNA), and the $\Delta\Delta C_T$ method was used to obtain the relative expression level for each gene. Data are shown as mean \pm S.D. Error bars were generated by transformation of mean $\Delta\Delta C_T$ values \pm one S.D. unit, taken to 2^{-n} . **A**, gene expression changes in 20 genes (5 per drug) detected by real time RT-PCR. **B**, validation of drug-specific gene expression responses. Primers specific for *ERG11* were assayed in real time PCRs using RNA from AmB-, CPF-, and 5-FC-treated samples. Primers specific for *RNR3* were tested against RNA from ketoconazole-, amphotericin B-, and caspofungin-treated samples. *KTZ*, ketoconazole; *AmB*, amphotericin B; *CPF*, caspofungin.



list of genes that responded similarly to the four drug treatments. Non-highlighted genes represent those genes that responded to at least three of the four drugs tested, and genes in boldface italic represent those that responded to all four drug treatments. This list probably represents genes that are involved in survival mechanisms that yeast cells employ when exposed to toxic agents. It is worth noting that only 24 genes responded similarly to three different drug exposures, indicating that the majority of responses are not related to generalized survival mechanisms and therefore represent specific responses to each drug. However, some of the genes shown in Table II are represented in the DI graphs used to identify drug-specific responses (Fig. 10). These genes include *GRE1*, *HSP12*, and *DDR2* (amphotericin B); *AGA1* and *YPS3* (caspofungin); and *MEP2* and *GAP1* (5-FC). It is worth noting that these genes were strongly induced or strongly repressed in response to specific drug treatments. For example, *MEP2*, encoding an NH_4^+ transport protein, is ~ 34 -fold repressed by 5-FC (Fig. 8), and this is possibly because of NH_4^+ accumulation in the cells due to 5-FC deamination. Similarly *GRE1*, a gene induced by osmotic stress, is ~ 93 -fold up-regulated by amphotericin B (Fig. 4); this up-regulation indicates the loss of osmotic balance resulting from the leakage of ions due to membrane damage. These results indicate that the magnitude of the fold change for a particular gene may be specific for a given treatment, even though its expression level may show changes under a variety of conditions as a generalized stress response. Thus, in these types of drug exposure studies, it is also important to examine the data using tools such as the difference index to identify specific *versus* nonspecific responses to drug treatments.

Validation of Microarray Data by Real Time RT-PCR—To validate the differential expression of genes obtained by microarray analysis, real time RT-PCR was performed using the same RNA from the original microarray experiment. Twenty genes (5 per drug) were selected not only to confirm their importance in the mechanism of action of the respective drugs (e.g. *ERG11* and *ERG26* for ketoconazole; *GSC2* for caspofungin; and *RNR3* and *RFA2* for 5-FC) but also to verify the novel responses identified in this work (e.g. *UPC2*, *DAN1*, and *AUS1* for ketoconazole; *PHM8*, *HXT5*, and *YPS3* for amphotericin B; *MTL1*, *RLM1*, *BAG7*, and *SLT2* for caspofungin; and *HO*,

tericin B (Fig. 4); this up-regulation indicates the loss of osmotic balance resulting from the leakage of ions due to membrane damage. These results indicate that the magnitude of the fold change for a particular gene may be specific for a given treatment, even though its expression level may show changes under a variety of conditions as a generalized stress response. Thus, in these types of drug exposure studies, it is also important to examine the data using tools such as the difference index to identify specific *versus* nonspecific responses to drug treatments.

DSE4, and *PCL1* for 5-FC). For all 20 genes, there was complete correlation between real time RT-PCR and microarray data (Fig. 11A). In agreement with the microarray data, 17 genes showed up-regulation and 3 genes showed down-regulation in response to drug treatment. Furthermore, genes that showed high levels of induction in the microarray experiment also showed high levels of induction in real time RT-PCR assays (e.g. *DAN1* in response to ketoconazole and *RNR3* in response to 5-FC). However, *DAN1* and *RNR3* showed a higher level of induction in real time RT-PCR assays (129- and 127-fold induction, respectively, Fig. 11A) compared with the microarray data (54- and 47-fold induction, respectively, Figs. 2 and 8). This discrepancy could be attributed to the fact that the Affymetrix software assigned an “absent” call to both genes in the untreated sample, i.e. these genes were undetectable in the untreated samples and were highly induced upon drug treatment. Fold induction values obtained from microarray analysis can be particularly misleading in cases where poor or no hybridization signals are generated in one of the samples due to low expression levels of specific genes in that sample.

For the remaining 18 genes, the levels of gene induction did not differ markedly between microarray data and real time RT-PCR data. A few minor discrepancies that were observed could be explained, in part, by the greater dynamic range of real time RT-PCR assays. In addition, cross-hybridization may occur in microarray experiments with splice variants or related genes. For example, *HXT5* shows 65–75% identity at the nucleotide level to 17 related *HXT* genes.

In order to provide further confirmation of gene-specific responses to the drugs tested, additional negative control assays were performed. Primers specific for the *ERG11* gene were used in real time RT-PCR assays with RNA from amphotericin B-, caspofungin-, and 5-FC-treated samples. For all three samples, there was no significant induction of *ERG11* compared with the untreated control (Fig. 11B). Similarly, primers specific for the *RNR3* gene were tested with RNA from ketoconazole-, amphotericin B-, and caspofungin-treated samples. No significant induction of *RNR3* was detected in the three samples assayed (Fig. 11B). These results provide further evidence that the genes assayed by real time RT-PCR respond specifically to the respective drug treatments.

Susceptibility of *upc2* Mutant Strains to Ketoconazole—Because ketoconazole treatment resulted in the induction of *UPC2* (Fig. 2), a gene that encodes a transcription factor involved in regulating sterol biosynthesis and uptake, it is possible that any alteration in *UPC2* levels would have an effect on the susceptibility to ketoconazole. The *upc2-1* mutant accumulates ~6-fold more exogenous sterols compared with the wild type (39), which could reduce the level of inhibition caused by ketoconazole. Conversely, the deletion of *UPC2* results in reduced sterol uptake (39, 72) and could cause an increase in the inhibitory effect of ketoconazole. To test this hypothesis, the susceptibility of *S. cerevisiae* to ketoconazole was tested in the *upc2-1* mutant and a *upc2* deletion mutant.

Experiments were performed in the presence of ergosterol (10 μ g/ml), and cells were grown under anaerobic conditions to allow exogenous sterol uptake. It is known that *S. cerevisiae* cells are not able to uptake sterols under aerobic conditions due to the phenomenon of aerobic sterol exclusion, i.e. under aerobic conditions *S. cerevisiae* cells synthesize ergosterol and are not dependent upon exogenous sterols for survival (73).

As can be seen in Fig. 12, the *upc2-1* mutant was less susceptible to ketoconazole under these growth conditions. The IC_{50} for ketoconazole increased from 2 μ g/ml in the wild type strain to 4.8 μ g/ml in the *upc2-1* mutant, and the MIC (concentration at which there was no detectable growth) increased

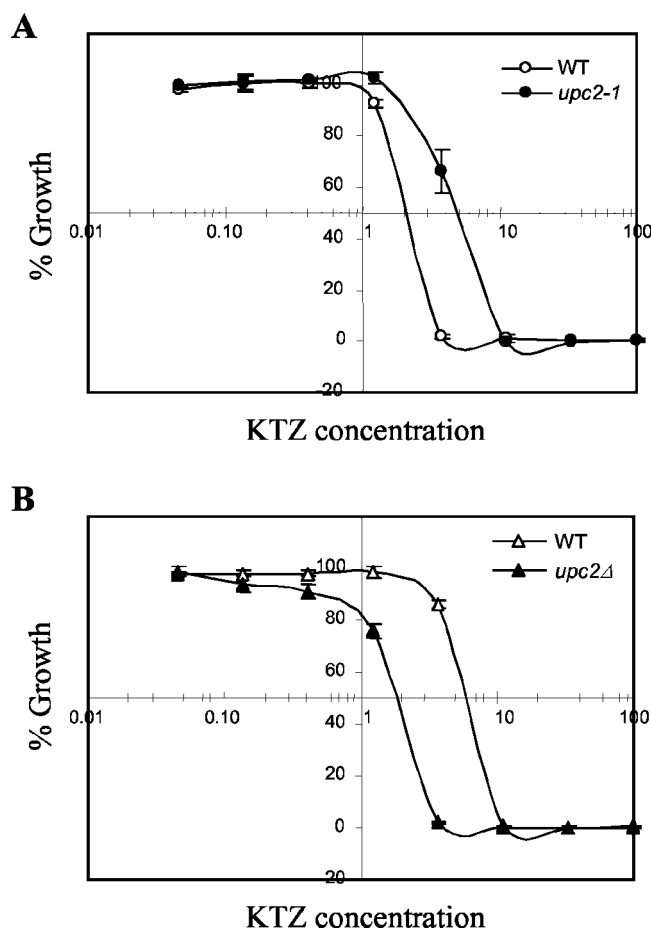


FIG. 12. Dose-response curves of ketoconazole-treated cells. Mutant strains and their isogenic wild type strains were grown in microplates, at 30 °C for 48 h, in SC medium containing varying concentrations of ketoconazole and 10 μ g/ml ergosterol. Microplates were incubated under anaerobic conditions as described under “Experimental Procedures.” Four replicates of each assay were performed. Percent growth is shown as mean \pm S.D. A, susceptibility to ketoconazole of *upc2-1* mutant and its isogenic wild type (WT). B, susceptibility to ketoconazole of *upc2Δ* mutant and its isogenic wild type. KTZ, ketoconazole.

from 3.7 to 11.1 μ g/ml, respectively (Fig. 12A). No significant difference in ketoconazole susceptibility was detected under aerobic growth conditions even though the *upc2-1* mutant is capable of accumulating exogenous sterols during aerobic growth (data not shown). It is possible that the amount of sterol accumulated in the *upc2-1* mutant under aerobic conditions is not enough to counterbalance the effect of ketoconazole. Because *UPC2* is induced under anaerobic conditions (74, 75), it is likely that higher levels of sterol are accumulated anaerobically resulting in reduced susceptibility to ketoconazole.

Comparison of the *upc2Δ* mutant to its isogenic wild type indicated that the *upc2Δ* mutant was more susceptible to ketoconazole under the experimental conditions tested (Fig. 12B). The IC_{50} decreased from 6 μ g/ml in the wild type strain to 1.8 μ g/ml in the *upc2Δ* strain, and the MIC decreased from 11.1 to 3.7 μ g/ml, respectively. The increased susceptibility to ketoconazole of the *upc2Δ* mutant under anaerobic conditions is consistent with the hypothesis that reduced sterol uptake in the mutant would increase the inhibitory effect of ketoconazole.

Interestingly, a recent report (21) indicated that *UPC2* was induced in *S. cerevisiae* cells exhibiting reduced susceptibility to fluconazole and itraconazole. Similar results were reported in *S. cerevisiae* cells overexpressing the *SUT1* gene (encoding a second transcription factor involved in regulating sterol up-

take), which showed increased resistance to the ergosterol biosynthesis inhibitor fenpropimorph, in medium supplemented with ergosterol (24). Taken together, the current study suggests an important role for *UPC2* in determining sensitivity to ergosterol biosynthesis inhibitors.

In conclusion, the microarray studies reported here have revealed specific changes in gene expression consistent with known mechanisms of action, changes suggesting previously unidentified effects of these agents, and nonspecific changes to antifungal agents in general. Gene expression profiling will likely be useful for screening compounds to identify candidates with specific mechanisms of antifungal activity. The approach will also be useful in identifying the mechanisms of action for compounds identified as exhibiting activity through other screens. These findings lay the groundwork for the application of functional genomics to identify gene expression profiles that will be useful in an antifungal drug development program.

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